THE EXPERIMENTAL INDUCTION OF PRENATAL MORTALITY AND THE SUBSEQUENT ELIMINATION OF THE DEAD EMBRYOS IN RABBITS

BY F. W. ROGERS BRAMBELL, MEGAN HENDERSON AND IVOR H. MILLS

From the Department of Zoology, University College of North Wales, Bangor

(*Received* 9 *November* 1947)

(With Two Text-figures)

INTRODUCTION

Extensive prenatal mortality occurs in wild rabbits after implantation. All the embryos die and are reabsorbed in many of the litters between the tenth and fifteenth days post-coitum (Brambell, 1942, 1944). Whereas the proportion of dead embryos in surviving litters could be calculated directly, the proportion of litters represented by those with all the embryos reabsorbing, which have consequently ceased to develop, could not be estimated without knowing the duration of the reabsorptive processes. It was essential to know also whether the dead embryos were always reabsorbed or whether they might be aborted and, if so, the factors which determine whether reabsorption or abortion occurs. Search of the literature failed to provide adequate information on these points and the present work was undertaken in consequence. The object of the work was confined to establishing the time relations of reabsorption and abortion in the rabbit. A review of the extensive literature on the endocrine aspect of the maintenance of pregnancy and any attempt to interpret the results from an endocrinological viewpoint have been avoided intentionally. The work has provided the material for histological investigation of the reabsorptive processes and of the condition of the uterus after abortion compared with that after parturition. These results will be published separately.

Mammalian embryos in the uterus have been killed experimentally by a variety of means. Giacomini (1893) and Hammond (1917) removed the embryos in rabbits surgically but allowed the placentae to remain in the uteri. Newton (1935) destroyed the embryos in the uteri of mice by manipulation and van Wagenen & Newton (1943) removed the embryos surgically in monkeys. Ovariectomy has been found by many authors to result in the termination of pregnancy in most, but not in all, species. Injection into the mother of various substances such as urea (Feis, 1894), colchicine (Kerr, 1947), sex-hormones including natural and artificial oestrogens (Parkes, Dodds & Noble, 1938), etc. have been employed. Huggett & Pritchard (1945), working on rats, employed four methods: direct surgical interference, ovariectomy, oestrone injections and gonadotrophic hormone injections.

It was necessary to know, first, the times from the death of an embryo rabbit until (*a*) fragmentation was so advanced that it was no longer possible to determine the age at death, and (*b*) the reabsorptive site in the uterus was no longer recognizable as such macroscopically; secondly, how these periods varied (*a*) with the age of the embryos at death, and (*b*) when all the embryos in the litter, or only some of them, were killed; thirdly, if abortion, rather than reabsorption, might occur. This involved killing, at will, either all the embryos simultaneously or else only selected ones; hence two contrasting methods were used, that of injection of synthetic oestrogen and that of direct surgical interference with the embryos.

MATERIAL AND TECHNIQUE

The material employed consisted of three wild rabbits and forty-nine tame rabbits; the majority of the latter belonged to the Agricultural Research Council's Compton strain of Dutch rabbit. The tame rabbits were permitted to copulate twice in rapid succession and only those which did so were employed. The stage of development of the embryos was dated from the time of the first copulation. The wild rabbits were freshly caught and were pregnant at the time of capture. They were part of a large sample of wild rabbits upon which exploratory laparotomies were performed to determine the stage of pregnancy.

A uniform dose of 5 mg. of stilboestrol in olive oil was injected subcutaneously in all the animals in which the embryos were killed by means of an oestrogen.

Laparotomy was performed under full ether anaesthesia and with appropriate aseptic precautions in those animals in which an attempt was made to destroy the embryos by surgical means. The method employed was to perforate the selected uterine swellings through the antimesometrial wall of the uterus, using a fine suture needle. The needle was passed into the swelling and several stabs at the embryo were made. It was possible as a rule to confirm that the needle passed through the embryo since the antimesometrial wall of the swelling at the stages employed is semi-transparent. Care was taken to ensure, so far as possible, that the needle passed through the head of the embryo. A very small number of animals died under anaesthesia, but otherwise no animals showed adverse after-effects.

The embryos were judged to be dead in all experiments when they had become flaccid and white. The heart was never beating in such embryos. These criteria were certainly rigorous so far as the embryo is concerned, since they entailed some measure of autolysis, but it does not follow that the embryonic placental tissues had died simultaneously. The stage of development attained by embryos already dead at autopsy was taken to represent the age at which they had died.

Since material was required for the histological investigation of the course of reabsorption in each experimental series, some of the swellings were unopened at autopsy and were preserved intact.

EXPERIMENTS

Destruction of embryos by injection of stilboestrol

Three series of experiments were performed in which stilboestrol was injected at 11, 15¾ and 19–20 days post-coitum respectively. No embryos survived the injection by more than 24 hr., judged by the stage of development attained by the embryos as determined by comparison with the normal table of Minot & Taylor (1905).

The first series consisted of fifteen rabbits, of which one proved to be non-

pregnant and was rejected. Particulars of the animals, which were killed and examined at intervals ranging from 1 to 10 days after injection at 11 days, are given in Table 1. Four days after injection, or 3–3½ days after death, appears to be the limit at which the age can be determined. The reabsorption sites were barely distinguishable from old placental sites at 10 days after injection, and this may be taken as the limit at which they could be recognized, were it not known that reabsorption, rather than abortion or parturition, had occurred. The progressive decrease in diameter of the swellings after the death of the embryos is remarkably uniform. The data are represented graphically, together with the data of the diameters of the uteri between swellings, in Fig. 1. The diameters of the swellings fall about a line which would intersect that of the diameters of the uteri on the 10th day.

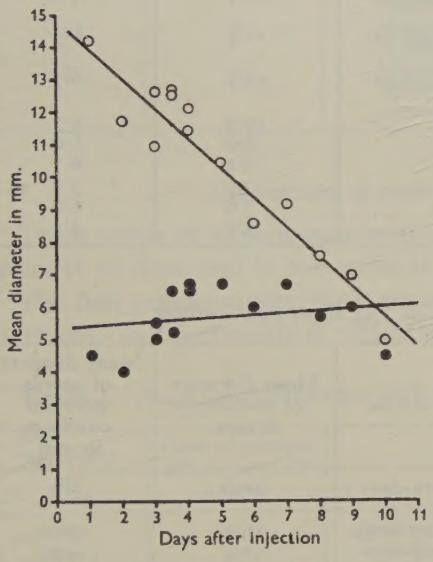


Fig. 1

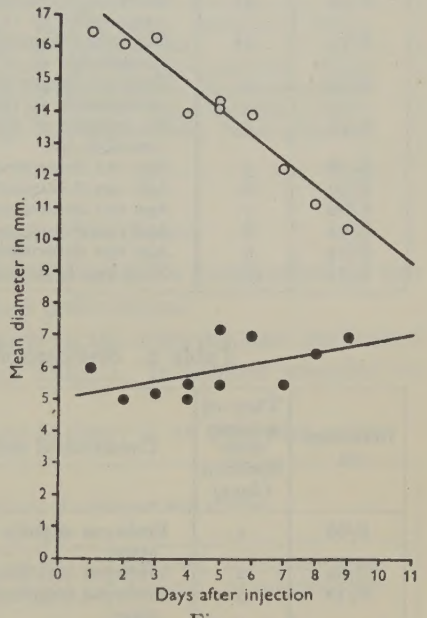


Fig. 2

Figs. 1 and 2. Progressive decrease in diameter of the conceptuses after the death of the embryos. The hollow circles represent the mean diameters of the swellings and the solid circles the mean diameters of the uterus between swellings. The fitted regression lines are shown.

The second series consisted of thirteen animals injected at 15¾ days and one at 17 days post-coitum. One was excluded as the embryos had evidently begun to retrogress several days before the injection. Particulars of the remainder, which were killed and examined at intervals of from 1 to 9 days after injection, are given in Table 2. Five days after injection, or 4–4½ days after death, was the maximum time at which the stage of development of the embryos could be determined. The 9th day was the limit at which the reabsorption sites could be recognized as such, macroscopically, since they were scarcely distinguishable from old placental sites at that time, but they remain visible as slight swellings on the uterus for some days longer. The rate of decrease in diameter of the swellings can be seen from Fig. 2,

Table 1. *Stilboestrol injection at 11 days post-coitum*

Reference no.	Time of autopsy after injection (days)	Condition of embryos and uterus	Mean diameter of swellings in mm.	Mean diameter of uterus between swellings in mm.
E/52	1	Embryos slightly limp. At 11½-day-stage	14·3	4·5
E/53	2	Embryos limp. At 11½-day-stage	11·8	4·0
E/54	3	Embryos very limp and autolysing. At 11½-day-stage	11·0	5·5
E/47	3	Embryos limp and slightly distorted. At 11½–12-day-stage	12·7	5·0
E/70	3½	Embryos fragmented. Age just determinable as 11½ days	12·7	6·5
E/71	3½	Embryos fragmented. Age just determinable as 12 days	12·7	5·2
E/50	4	Embryos fragmented. Age just determinable as 12 days	11·5	6·5
E/49	4	No embryonic fragments of determinable age	12·2	6·7
E/58	5	Age not determinable	10·5	6·7
E/59	6	Age not determinable	8·6	6·0
E/60	7	Age not determinable	9·2	6·7
E/72	8	Age not determinable	7·6	5·7
E/73	9	Age not determinable	7·0	6·0
E/74	10	Sites barely identifiable	5·0	4·5

Table 2. *Stilboestrol injection at 15¾ days post-coitum*

Reference no.	Time of autopsy after injection (days)	Condition of embryos and uterus	Mean diameter of swellings in mm.	Mean diameter of uterus between swellings in mm.
E/66	1	Embryos slightly limp. At 16-day-stage	16·6	6·0
E/62	2	Embryos very limp. At 16-day-stage	16·2	5·0
E/48	3	Embryos fragmenting. At 16½-day-stage	16·4	5·2
E/64	4	Embryos limp and distorted. At 16-day-stage	14·0	5·5
E/65	4	Embryo limp and distorted. At 16-day-stage	—	5·0
E/67	5	Embryos fragmented. At 17-day-stage	14·2	7·2
E/56	5	Age not determinable	14·4	5·5
E/63	6	Age not determinable	14·0	7·0
E/68	7	Age not determinable	12·3	5·5
E/57	8	Age not determinable	11·2	6·5
E/69	9	Sites hardly visible, externally	10·4	7·0
E/51*	5	Age not determinable	—	—

* (Injected at 17 days post-coitum.)

the principal difference from those killed at 11 days being in the larger size of the swellings at the time of death. Otherwise the chief difference from the previous series was the tendency for the placenta to become separated from the uterine wall. The separation had begun the day after injection but was not complete until the

5th day. The embryonic part of the placenta was not present on the 8th day and on the 9th day only the unhealed sites marked the former positions of the placentae.

The third series consisted of only three rabbits injected at 19–20 days post-coitum and is summarized in Table 3. All aborted, but, in one some of the embryos were retained and had regressed *in situ* at the time of autopsy, 9 days after injection. The other two aborted all the embryos on the 5th day after injection, one aborting all, and the other some, of the placentae as well.

Table 3. *Stilboestrol injection at* 19–20 *days post-coitum*

Reference no.	Time of autopsy after injection (days)	Condition of embryos and uterus
E/113	9	Appears to have aborted some embryos. Others reabsorbing. Age not determinable
E/91	6	Aborted embryos on 5th day after injection. Much autolysed and age not determinable. Placentae retained
E/92	9	Aborted embryos on 5th day after injection. Much autolysed and age not determinable. Some placentae retained

Destruction of embryos by surgical interference

Three series of experiments were performed, the embryos being killed in two series at 16 days, and in one series at 19–20 days post-coitum.

The first series consists of six animals in which all the embryos were perforated at 16 days and particulars of which are given in Table 4. In another intended for

Table 4. *Surgical interference with all embryos in litters at* 16 *days post-coitum*

Reference no.	Time of autopsy after operation (days)	Condition of embryos and uterus
E/85	5	Embryos intact but flabby and distorted. At 16½-day-stage
E/107	7	Embryos intact but flabby and distorted. At 16½-day-stage (one at 17 days)
E/123	8	Aborted all except one placenta. Sites unhealed
E/110	9	Aborted. Sites unhealed
E/86	10	No trace of embryos or placentae
E/83	11	Sites recognizable but small

this series, one embryo survived though the membranes had been perforated, and it was therefore transferred to the second series. The embryos were intact, though flabby and distorted, in the first two animals killed at 5 and 7 days after operation respectively. Hence the dissolution of the embryos in these was much slower than in those of the corresponding series injected with stilboestrol at 15¾ days post-coitum, in which no intact embryos were found more than 3½ days after death. The second of these animals (E/107) was plucking fur and nest-making when killed and the uterus was observed at autopsy to be contracting strongly. It is probable that this animal would have aborted soon if it had lived. The next two animals, killed at 7¾ and at 9 days after operation respectively, had aborted but the abortions were not

recovered for examination. The two remaining animals, killed at $9\frac{3}{4}$ and at 11 days after operation, had no trace of embryos or placentae and it is probable that they aborted some days previously.

The second series, in which some of the embryos were killed at 16 days post-coitum and some survived, includes eight tame rabbits. Two wild ones, in which partial hysterectomy was performed, and some of the embryos in the remaining uterus were killed, can be included, as the estimated age of the embryos at operation was 15 and 17 days respectively. Particulars are given in Table 5. One (E/84)

Table 5. *Surgical interference with some embryos in litters at 16 days post-coitum*

Reference no.	Time of autopsy after operation (days)	Condition of embryos and uterus
E/87	0	Died during operation
E/84	4	1 R., 2 L. and 4 L. perforated. 2 L. died at $16\frac{1}{2}$–17 days, fragmenting. 1 L. died at 18 days, fragmenting. Three embryos survived and were aborted at 19–$19\frac{1}{2}$ days
E/122	6	All embryos perforated. One embryo survived until autopsy at 22 days. Remainder died at $16\frac{1}{2}$ days, were intact and age just determinable
E/98	10	2 L., 2 R. and 4 R. perforated at operation. 2 L., 2 R., 3 R. and 4 R. died at 16–$16\frac{1}{2}$ days, intact but distorted. 6 R. reabsorbing. Three embryos survived until autopsy at 26 days
E/82	13	2 R., 2 L. and 4 L. perforated. 2 L. and 4 L. survived. 2 R. died at $16\frac{1}{2}$ days, age still determinable. Eight embryos survived until autopsy at 29 days
E/89	13	2 R., 2 L. and 4 L. perforated. 2 R., 3 L. and 4 L. died at 16 days, fragmenting but age determinable. 1 L. and 2 L. died at 17–18 days. Three embryos survived until autopsy at 29 days
E/80	13	2 R. and 2 L. perforated, but all embryos survived until autopsy at 29 days
E/81	13	1 R. perforated at operation, but survived until approx. 26 days. 1 L. reabsorbing. One embryo survived until autopsy at 29 days
W/14	5	1 L. removed at operation. 2 R. perforated, died at 15–$15\frac{1}{2}$ days, limp but intact. Two embryos survived until autopsy at 20 days
W/29	12	1 R. removed at operation. 1 L. perforated, died at 17–$17\frac{1}{2}$ days, flabby and distorted, but intact. Two embryos survived until autopsy at 29 days

aborted the surviving embryos 3 days after operation and was killed as soon as this was discovered. Six were successful experiments in that some of the embryos survived and some died, although some of the embryos which died had not been perforated and some which were damaged survived. The dead embryos in all of these were recognizable, and their age at death could be determined. The dead embryos in most cases were intact, though flaccid and distorted, even when the animal was not killed until near full term, upwards of 12 days after operation.

The third series consists of four tame rabbits in which all the embryos were perforated at 19–20 days post-coitum and one wild one in which one embryo only was perforated. The results are summarized in Table 6. Two of the embryos in the perforated swellings survived in one tame animal (E/114). Two of the tame animals aborted all the embryos and placentae. The aborted embryos were recovered and

had died at the time of operation in one of these (E/115), which aborted 3 days after operation. The embryos were not recovered and the precise time of abortion was unknown in the other (E/120), killed 8 days after operation. The condition of the uterus of the wild rabbit (W/22), when killed at 8 days after operation, was inconsistent with reabsorption and it must have aborted soon after the operation as the placental sites were healed. The remaining tame rabbit (E/121) was reabsorbing all the embryos, which were still intact, and at the 20-day-stage, when it was killed 8 days after operation. This is the only animal in which all the embryos were killed at 20 days, either by stilboestrol or surgically, and which did not abort.

Table 6. *Surgical interference with all embryos in litters at 19–20 days post-coitum*

Reference no.	Time of autopsy after operation (days)	Condition of embryos and uterus
E/114	5	Two embryos survived until autopsy at 25 days. Three embryos died at 20 days, flabby and distorted, but intact
E/115	4	Aborted embryos and placentae at 23 days. Embryos died at 20 days, flabby and distorted
E/120	8	Aborted embryos and placentae. One embryo reabsorbing at time of operation. Others punctured. No remains of determinable age recovered
E/121	8	Reabsorbing. All embryos punctured, died at 20 days. All present, intact with placentae
W/22	8	Probably aborted. 1 L. only punctured. Healed sites remained at autopsy

CONCLUSIONS

The results show that 5 mg. of stilboestrol administered subcutaneously kill all the embryos with uniformity, whether at $11\frac{1}{2}$, 16 or 20 days post-coitum and within 24 hr. of administration. Laparotomy and puncture of the swellings is by no means so effective. It was anticipated that the mere puncture of the membranes, with the consequent loss of some of the embryonic fluid contained by them, would be sufficient to kill the embryos. The fact that many survived loss of fluid, and some probably direct damage as well, was surprising.

The speed at which reabsorption proceeds, when it occurs, varies widely according to the age of the embryos at death, the method of killing and whether some or all in the litter die. The remains of the embryos permit determination of the age at death for at most $3–3\frac{1}{2}$ days after death at $11\frac{1}{2}$ days, as compared with $4–4\frac{1}{2}$ days after death at 16 days post-coitum, when killed by stilboestrol. The reabsorption sites remain recognizable as such macroscopically for 9–10 days after death from stilboestrol administration at either 11 or $15\frac{3}{4}$ days post-coitum; although the sites remain visible after that time, especially in the $15\frac{3}{4}$-day-series, it is not then possible to distinguish them from old post-partum placental sites. The embryos remain intact and their age at death can be determined for at least 7 days after death at

16 days, and 8 days after death at 20 days when all have been killed surgically. Hence autolysis of the embryos must proceed much more quickly after stilboestrol administration than after surgical interference. The age of the embryos at death can be determined, and they often remain intact for at least 13 days after death at 16 days post-coitum, that is until full term, when some of the other embryos in the litter survive. Obviously in this case they are very much more persistent than when all are killed with stilboestrol. They are also very much more persistent than when all are killed surgically but the evidence is inconclusive as to whether this is due to slower autolysis or to reabsorption not being curtailed by subsequent abortion, as it often is when all the embryos are dead.

The method of removal of dead embryos and membranes appears to vary according to the stage of development at which death occurs and to whether or not some of the embryos survive. It may vary also according to the manner in which the embryos are killed. Reabsorption occurred in every case where the embryos were killed by stilboestrol at $11\frac{1}{2}$ or 16 days. The results, when all the embryos were killed surgically at 16 days, were consistent with an initial period of reabsorption lasting 7 days being followed by abortion of the remnants. Conversely when some only of the embryos were killed surgically at 16 days reabsorption was the rule, one animal only aborting, and that incompletely. Although the number of animals in which the embryos were killed at 20 days was small it is significant that all three of those injected with stilboestrol aborted, as did two certainly, and probably three, of those in which the embryos were killed surgically. One in which all the embryos were killed and one in which some survived, reabsorbed the dead embryos and did not abort.

The difference in the methods of removal between the series in which all the embryos were killed at 16 days by stilboestrol and by perforation respectively is remarkable. It was observed that, in the stilboestrol series, although the embryos and placentae always remained *in situ*, the placentae tended to become detached through the development of clefts in the connective tissue of the decidua basalis close to the muscularis. Although this tendency was apparent soon after the death of the embryos complete separation of the placentae was not observed until 5 days after injection. Probably abortion cannot occur until this zone of weakness has differentiated. The appearances observed suggested that although the placental tissues died rapidly after stilboestrol injection they did not do so after perforation, and often survived the death of the embryos, so reaching a stage of development in advance of that at which the embryos died. This is not improbable since many authors, including Newton (1935), van Wagenen & Newton (1943) and Huggett & Pritchard (1945), have shown that the placenta can survive the death of the embryo and may even persist until normal full term in several species. Thus the difference between the two 16-day-series described herein, in that abortion did not occur in one and was frequent in the other, may depend on a difference in the stage of development attained by the placentae, resulting from the difference in the methods of killing the embryos.

It is difficult to determine the time at which abortion occurs because rabbits eat

the aborted embryos and membranes immediately. It was known accurately in four of the eleven cases in which abortion was believed to have occurred. These four cases vary from 19½ to 25 days post-coitum. The probable time of abortion in the others could be estimated from the known time of death of the embryos and the condition of the placental sites at autopsy. All the estimates are consistent with it having occurred between 20 and 25 days post-coitum.

SUMMARY

1. All the embryos in utero in pregnant rabbits were killed in a few hours by 5 mg. of stilboestrol administered subcutaneously. Experiments were performed on fourteen animals at 11 days, twelve animals at 15¾ days and three animals at 19–20 days post-coitum.

2. Embryos killed with stilboestrol administered at 11 or 15¾ days post-coitum were invariably reabsorbed. Abortion occurred when the embryos were killed by stilboestrol at 19–20 days.

3. Some or all of the embryos were killed in sixteen animals at 16 days and in five animals at 19–20 days post-coitum by perforating the uterus, membranes and embryos with a needle at laparotomy. Many of the embryos survived this treatment.

4. Abortion occurs as a rule, but not invariably, when all the embryos are killed by surgical means. In the 16-day-series abortion is probably preceded by an initial period of autolysis. In the 20-day-series one animal reabsorbed but the others aborted.

5. Destruction of some only of the embryos at 16 or 20 days post-coitum by surgical means usually results in reabsorption of the dead embryos and the maintenance of pregnancy, but may result in abortion.

6. The occurrence of abortion may depend on the stage of development attained by the placenta.

7. Abortion occurred at 19½–25 days post-coitum. There was no evidence that abortion occurred before the 19th day.

8. The speed at which reabsorption proceeds varies with the stage of development of the embryos at the time of death, the manner of death, and whether some or all the embryos die. Reabsorption proceeds much more quickly after stilboestrol administration than after surgical interference. The dead embryos are much more persistent when others survive and the mother remains pregnant than when all are killed surgically. It is uncertain whether the greater rapidity of removal, when all embryos die, is due to more rapid autolysis or is due to the curtailment of reabsorption by abortion.

We wish to thank Miss Patricia Allen for much assistance in the conduct of the experiments. We are indebted to Dr A. S. Parkes, F.R.S., for suggesting the use of stilboestrol as a convenient means of killing the embryos in the intact pregnant rabbit. We are indebted to Sir Michael Duff, Bart., for the supplies of live wild rabbits and we wish to acknowledge the interest and care his Head Keeper,

Mr Charles Parker, took in obtaining the animals in satisfactory condition. We wish to acknowledge the technical assistance of Mr R. A. Lansdown and Mr W. Holland. This work is part of a scheme of research on prenatal mortality supported by the Agricultural Research Council, to whom we are greatly indebted.

REFERENCES

BRAMBELL, F. W. ROGERS (1942). *Proc. Roy. Soc.* B, **130**, 462–79.
BRAMBELL, F. W. ROGERS (1944). *Proc. zool. Soc., Lond.*, **114**, 1–45.
FEIS, W. (1894). *Arch. Gynaek.* **46**, 147.
GIACOMINI, C. (1893). *Arch. ital. de biol.* **18**, 400.
HAMMOND, J. (1917). *Proc. Roy. Soc.* B, **89**, 534–45.
HUGGETT, A. G. & PRITCHARD, J. J. (1945). *Proc. Roy. Soc. Med.* **38**, 261–71.
KERR, T. (1947). *Proc. zool. Soc., Lond.*, **116**, 551–64.
MINOT, C. S. & TAYLOR, E. (1905). *Normentaf. Wirbelt.* **5**.
NEWTON, W. H. (1935). *J. Physiol.* **84**, 196–207.
PARKES, A. S., DODDS, E. C. & NOBLE, R. L. (1938). *Brit. Med. J.* **2**, 557–9.
VAN WAGENEN, G. & NEWTON, W. H. (1943). *Surg. Gynaec. Obstet.* **77**, 539–43.

THE ACTIVITY OF RAM SPERMATOZOA

By LORD ROTHSCHILD

From the Department of Zoology, Cambridge

(*Received* 6 *January* 1948)

(With Plate 4 and Four Text-figures)

INTRODUCTION

The object of this paper is to describe a possible new method of measuring the activity of ram spermatozoa. The existing methods may be classified as follows:

Visual estimation of motility, under the microscope.

Manometric determination of O_2 consumption or glycolysis.

Measurement of dehydrogenase activity.

Measurement of fructolytic activity (Mann, 1946; 1948).

Measurement of pH changes (Laing, 1945).

The limitations of the first method, due to its subjective and relatively unquantitative nature, are obvious. The other methods also suffer from certain disadvantages. Undiluted semen is not suitable for manometric experiments, while its dilution may introduce complications unless certain precautions are taken (Tosic & Walton, 1947); moreover there is no *a priori* reason for assuming that O_2 consumption is a direct measure of motility, particularly as immobile spermatozoa consume oxygen (Gray, 1928) and spermatozoa are motile in anaerobic conditions. Anaerobic CO_2 production may be a measure of acid production, but proof that glycolysis is directly proportional to motility depends on comparing CO_2 or acid production *data* with some other quantitative, but unrelated, method of assessing sperm motility. Lardy & Phillips (1941) showed that bull spermatozoa retain their motility for some time when glycolytic and oxidative mechanisms are blocked simultaneously, which may cast doubt on the value of oxidation or glycolysis measurements as indicators of sperm motility. For similar reasons, some misgivings might be entertained about the methylene-blue reduction test (dehydrogenase activity). All these methods involve the removal of samples from the original semen specimen: all, except visual estimation of motility, involve measurements or manipulations which cause a fairly considerable delay before a motility assessment is available.

Active ram semen exhibits a striking phenomenon, sometimes known as 'wave formation', which is due to the spontaneous reversible aggregation of spermatozoa in the suspension. These aggregations form and disrupt throughout the semen, while the spermatozoa are active. It seemed possible that such macroscopic changes in the 'structure' of the sperm suspension might be associated with variations in its electrical properties.

The experiments recorded in this paper are a description of electrical changes observed in suspensions of ram spermatozoa. Their relationship with the 'waves' referred to above, and the relationship of the waves with sperm motility, are discussed in detail later.

Throughout this paper, such phrases as 'active spermatozoa', 'feebly active spermatozoa', and 'intensity of waves' will be found. The fact that these expressions, which reflect an unsatisfactory situation in the field of sperm physiology, must still be used, is one of the reasons for doing these electrical experiments.

MATERIAL

Ram semen was supplied from the Animal Research Station, Cambridge, at 15° C. This was placed in a water bath at 37° C., the temperature at which experiments were done.

APPARATUS

Electrical measurements were made by placing the semen in a conductivity cell in the unknown arm of an a.c. bridge. The cell was an ordinary test-tube, with two platinized platinum electrodes dipping into the semen in the test-tube. The bridge was of conventional design, except that the detector was an oscilloscope instead of the more usual telephones. The bridge was energized by an oscillator working at 5000 cyc. and its sensitivity was such that a 0·01 % change in the unknown arm resistance could be detected without difficulty.

EXPERIMENTAL PROCEDURE

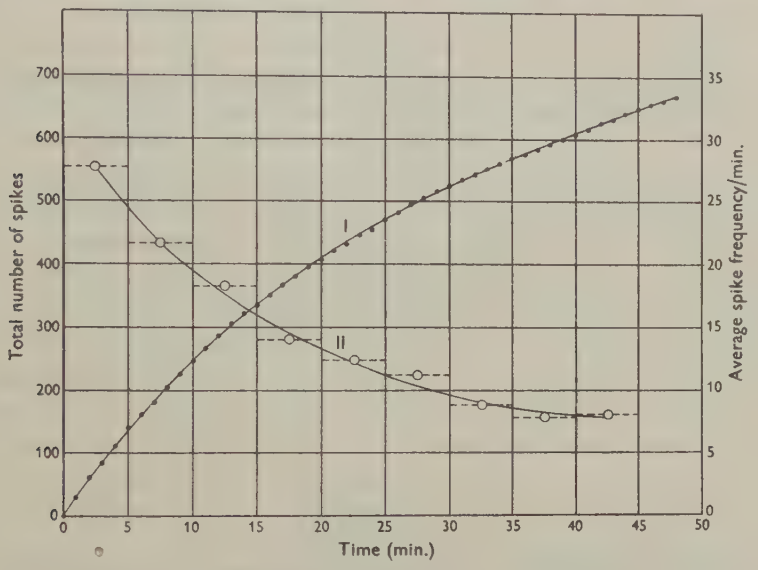
The sperm suspension in the unknown arm of the bridge is approximately balanced by particular values of the resistances and condensers in the standard arm. If the electrical properties of the unknown arm remain constant, the bridge will remain balanced. If not, the form and frequency of any deviations from balance, i.e. impedance changes, can be observed or photographed. Provided that we are interested in the frequency rather than the precise wave-form of any impedance changes that may occur, it is convenient to study such changes unilaterally in the envelope curve; that is, just outside the region of bridge balance. The advantages and difficulties in doing this have been discussed elsewhere (Hubbard & Rothschild, 1939).

RESULTS

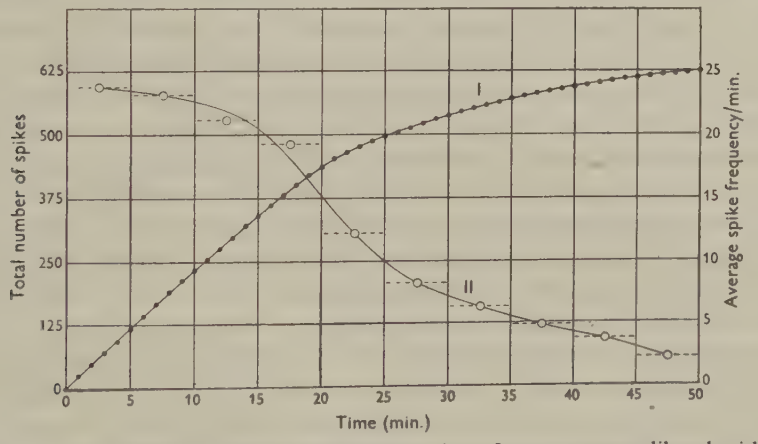
Absence of impedance changes in inactive and diluted suspensions. Plate 4*a*, v is a bridge record of a sperm suspension which exhibits no wave formation under the microscope. The electrical record shows no change in impedance and is, in effect, a straight line. Lack of wave formation may be caused by the spermatozoa being motionless or feebly active, which occurs in undiluted semen that has been kept at 37° C. for more than 60 min.; or it may be due to the semen having been diluted, for example, with glucose Ringer in the proportion of 9 parts of Ringer to 1 part of semen. The spermatozoa are very active in such a suspension, but no waves form.

Presence of impedance changes in active suspensions. Plate 4*a*, i–v is a bridge record of a fresh sample of undiluted semen exhibiting wave formation. It will be seen that

there are 12·5 spikes during the first minute and that the frequency of the spikes declines until after 150 min. they have disappeared altogether. Text-fig. 1 shows the total number of spikes or impedance changes plotted against time, and the corresponding rate curve for a sample of undiluted semen. This sample was



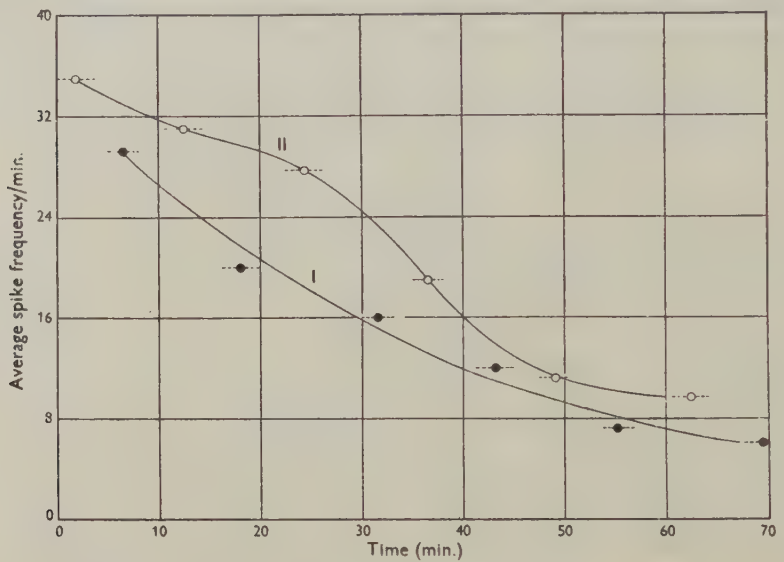
Text-fig. 1. *I*, total number of spikes plotted against time, for undiluted ram semen; *II*, average spike frequency/min., plotted against time, for the same suspension. The broken lines through each point show the time over which the frequency/min. was averaged.



Text-fig. 2. *I*, total number of spikes plotted against time, for ram semen diluted with an equal volume of isosmotic phosphate buffer; *II*, average spike frequency/min., plotted against time, for the same suspension. The broken lines through each point have the same significance as in Text-fig. 1.

markedly more active than the one whose electrical properties are shown in Plate 4*a*. The total number of spikes plotted against time and the corresponding rate curve for a sperm suspension diluted with an equal volume of isosmotic phosphate buffer are shown in Text-fig. 2.

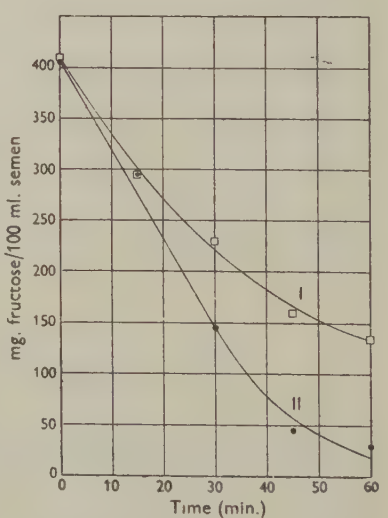
Effect of phosphate buffer. In Text-fig. 3, the variations in rate of spike frequency in two different suspensions of ram spermatozoa are compared. In *I*, the semen was diluted with an equal volume of glucose-free Ringer; in *II*, another sample of the



Text-fig. 3. Average spike frequency/min. plotted against time: *I*, ram semen diluted with an equal volume of glucose-free Ringer; *II*, the same ram semen diluted with an equal volume of isosmotic phosphate buffer. The broken lines through each point have the same significance as in Text-fig. 1.

same semen was diluted with an equal volume of phosphate buffer. It is evident, from Text-figs. 1, 2, and 3, that the phosphate buffer has a 'protective' effect on the spermatozoa in that the high rate of spike frequency is maintained in it for longer periods than in undiluted semen or semen diluted with Ringer. The same phenomenon is observed if the disappearance of fructose from the seminal plasma is used as an index of activity (Text-fig. 4). It was, however, noted that the seminal fructose is used up *before* the impedance changes have stopped.

Effect of temperature changes. Plate 4*b*, i and ii are records of impedance changes in ram semen at 16·0 and 36·0° C. There is a marked increase in frequency with temperature, the observed Q_{10} being about 2. This figure is too low, because the same semen sample was used for both experiments and, by the time the low temperature run was carried out, the initial frequency had declined. There are certain practical difficulties in doing Q_{10}



Text-fig. 4. Fructose in seminal plasma plotted against time: *I*, ram semen diluted with an equal volume of glucose-free Ringer; *II*, the same ram semen diluted with an equal volume of isosmotic phosphate buffer.

experiments in a more satisfactory way, as they require a series of water baths maintained at different constant temperatures and possibly two or more bridges with the associated apparatus. Nevertheless, for reasons discussed later, such experiments might be important.

DISCUSSION

The nature of the electrical changes. There may be as many as 10^8 spermatozoa between the electrodes in an experiment. Though the orientation of the spermatozoa is by no means random in such a suspension, it is difficult to believe that the observed electrical changes are in any way similar to the intrinsic variations in potential or resistance found in brain cells, contracting muscle fibres, active nerve fibres, or in *Nitella* after stimulation. The changes are much more likely to be caused by variations in the *position* of the biological material in the measuring system (i.e. with respect to the electrodes) than by changes in the membranes of individual spermatozoa. A somewhat analogous situation exists when a trout or salmon egg is placed in a conductivity cell in an a.c. bridge (Rothschild, 1947*a*, *b*). The egg is electrically heterogeneous and undergoes spontaneous movements rather like those of a precessing top. Because of this movement and the electrical heterogeneity of the egg surface, there is a periodic variation in the 'resistance' encountered by an electric current passing through the egg. Consequently the electrical properties of the system egg in measuring apparatus undergo periodic changes and it was partly because of this effect in fish eggs that similar experiments were tried on ram spermatozoa.

The apparatus is very sensitive to vibrations and it therefore seemed possible that the impedance changes might be due to currents of seminal plasma, produced by the waves, flowing past the electrodes. *Mytilus* gill, whose cilia produce marked currents of sea-water, were placed between the electrodes but no impedance changes were observed.

Can the periodic formation of sperm aggregations be responsible for the observed impedance changes? Provided that the volume concentration of spermatozoa in the seminal plasma is not too high, it is known from Maxwell's researches on the resistance of suspensions of spheres that the mere alteration in the packing of the spheres will have no effect on the resistance of the suspension, if the total volume concentration remains constant, which is so in the case under consideration. Maxwell's argument would not hold good if, at the same time as aggregation, there were some preferred orientation of the spermatozoa in the aggregations, coupled with individual electrical anisotropy. Ram spermatozoa are almost certain to be electrically anisotropic because of their shape, and in particular because of the shape of the sperm head, which resembles an elliptical disk. When examined in polarized light, it becomes evident that there is a great deal of orientation throughout the suspension and that it is not restricted entirely to the areas of aggregated spermatozoa.

The case for there being a correlation between the impedance changes and the waves would be made stronger if a strict correlation between the frequency of wave

formation and the frequency of the impedance changes could be found. The difficulty, if not the impossibility, of doing this is almost inherent in the problem. All that can be said at present is that when the waves form frequently, the impedance changes are frequent; when the waves form infrequently, the impedance changes are less frequent; and when there are no waves, there are no impedance changes. As a working hypothesis, it seems reasonable to assume that there is a correlation between the frequency of the waves and the frequency of the impedance changes; but there is as-yet no proof that this hypothesis is correct.

Correlation between sperm motility and frequency of wave formation. A high degree of motility is generally agreed to be necessary for successful fertilization. The following quotation, which refers to ram semen, from Anderson's *The Semen of Animals and its Use for Artificial Insemination* (1945, p. 129), is of interest in this connexion: 'Undiluted semen, examined microscopically, should show characteristic turbulent wave movements. Only ejaculates which show this very active motility should be used for insemination.' The writer evidently considers that there is a strict correlation between the degree of wave formation in the suspension and sperm motility, a view which is shared by Dr A. Walton of the Animal Research Station, Cambridge, and Mr L. E. A. Rowson of the Artificial Insemination Centre, Cambridge. The aggregations are not due to local deficiencies of O_2 as in certain protozoan cultures (Munro Fox, 1921), as they occur in anaerobic conditions. Nor is it likely that they are caused by local accumulations of CO_2, the explanation put forward by Lillie (1913) for the spontaneous reversible aggregations of *Nereis* spermatozoa. This explanation must probably be excluded, because wave formation persists when the evolved CO_2 is removed by KOH. There can be little doubt that the views expressed by the above-mentioned authorities are correct and that wave formation depends on motility, but also of course on density. In other words it is a function of collision frequency or the mean free path of each spermatozoon. This could be further investigated by observing the effect of *increasing* the density of the suspension on the frequency of the waves. There may be a physical explanation of these aggregations, given that, in certain circumstances, sperm heads stick together or remain contiguous. This possibility is not discussed because it is entirely hypothetical; it may, however, be mentioned that one difficulty to be resolved is that the waves often move through the suspension in a direction which tends to be at right angles to the long axis of the wave.

Correlation between impedance changes and sperm motility. If it is agreed that, on general grounds, there is a reasonable case for the hypothesis

Sperm motility → Wave formation → Impedance changes

for a given sperm density, the experiments described earlier on have a certain significance in confirming the hypothesis. When a suspension is fresh, the spermatozoa are very active; consequently they use up the fructose in the seminal plasma at a high rate. The lactic acid they produce has an inhibitory effect on their motility unless the suspension is adequately buffered. As the lactic acid exerts its effect, the rate of fructose disappearance will decrease. The fructolysis curves support this

interpretation and the impedance rate curves show the same characteristics. If, however, the semen is buffered with phosphate, we should expect the fructose to disappear more quickly, owing to neutralization of the lactic acid inhibition; but, for the same reason, the frequency of the impedance changes should fall more slowly than in unbuffered semen. The phosphate may of course have other functions in the metabolism of the spermatozoa, apart from acting as a buffer. The fact that the seminal fructose is used up before the impedance changes have ceased may be due to the experimental conditions not being completely anaerobic,* in which case lactate might be utilized as a substrate. On the other hand, it may reveal that fructolysis estimations are not an unequivocal index of motility in that after all the external fructose has been used up, some other substrate in the seminal plasma, or endogenous metabolic processes, may provide the energy for motility.

Possible applications of impedance measurements to determine sperm motility. Any suggestion that the frequency of the impedance changes in ram semen can be used as a quantitative measure of sperm motility and density would be premature. A number of comparative tests between the electrical measurements and the other existing methods of estimating sperm motility are necessary. Apart from this, the results so far obtained suggest further experiments of an electrical nature. Among the more important of these is to determine the effect of varying electrode size and separation on the observed impedance changes. A further experiment of interest would be to reduce the motility of the spermatozoa artificially, either by chemical (e.g. NaF) or physical means, and examine the results of such treatment on the impedance changes. The marked effect of changes in temperature on the frequency of the impedance changes is suggestive in this connexion.

An electrical method of assessing sperm motility would have certain advantages over the other methods described in outline earlier on. The advantages are:

(1) Measurements can be made on the actual semen obtained in the artificial vagina. No samples need be taken from the original one.

(2) Measurements can be made in a matter of minutes.

(3) The method is quantitative and non-subjective.

(4) Permanent photographic records can be kept.

(5) The method may provide a standard with which other methods of assessing sperm activity can be compared.

The main disadvantage of the method lies in the fact that its applicability depends on a hypothesis, for which at present there is only partial justification, that the impedance changes are due to periodic sperm aggregation and that the frequency of the latter is a direct measure of sperm motility, for a given density of sperm. The density factor is responsible for another disadvantage. The method could not be used to measure the activity of human, boar, or stallion spermatozoa unless these were concentrated to the required density by centrifugation. Centrifugation has disadvantages in these cases, apart from vitiating some of the advantages claimed for the method earlier on.

* Ram's semen is so viscous that, in the conditions of these experiments, the sperm between the electrodes must be in a virtually O_2-free medium.

SUMMARY

1. Concentrated suspensions of active ram spermatozoa exhibit periodic changes in their electrical properties when these are measured in an a.c. bridge.

2. Suspensions containing immobile or feebly active spermatozoa do not exhibit this phenomenon.

3. The frequency of the changes has the following characteristics:

(*a*) It is maximal when the suspension is fresh, and declines to zero after some 60 min. at 37° C.

(*b*) It can be maintained for about 20 min. at a high level by the addition of phosphate buffer.

(*c*) It is susceptible to changes in temperature, the Q_{10} between 16 and 36° C. being about 2.

4. A tentative hypothesis is put forward that these electrical changes are caused by the spontaneous, reversible aggregation, or 'turbulent wave formation', which is characteristic of active and concentrated suspensions of ram spermatozoa; and that they are due to the position of the spermatozoa in the measuring apparatus rather than to any intrinsic changes in the electrical properties of individual spermatozoa.

5. As the frequency and intensity of the wave formations are thought to be proportional to sperm motility, there is a possibility that the electrical changes might be used as an objective and physical measure of sperm motility.

6. Certain advantages and disadvantages in measuring the activity of the sperm suspensions by this method are discussed.

I am indebted to Dr A. Walton and the Staff of the Animal Research Station, Cambridge, for supplying the material for these experiments. It is also a pleasure to acknowledge much helpful advice from Dr Walton and Dr T. Mann of the Molteno Institute, Cambridge, who carried out the fructose estimations.

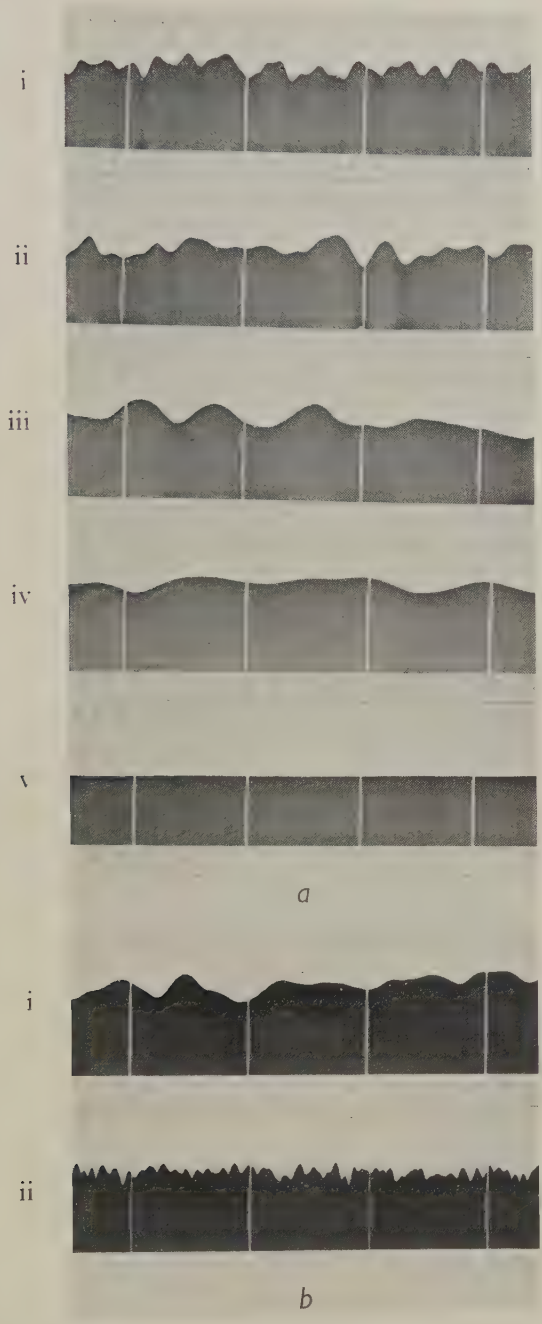
REFERENCES

ANDERSON, J. (1945). *The Semen of Animals and its use for Artificial Insemination.* Imperial Bureau of Animal Breeding and Genetics.
GRAY, J. (1928). *J. Exp. Biol.* **4**, 345.
HUBBARD, M. J. & ROTHSCHILD, LORD (1939). *Proc. Roy. Soc.* B, **127**, 510.
LAING, J. A. (1945). *J. Agric. Sci.* **35**, 1.
LARDY, H. A. & PHILLIPS, P. H. (1941). *J. Biol. Chem.* **138**, 195.
LILLIE, F. R. (1913). *J. Exp. Zool.* **14**, 515.
MANN, T. (1946). *Biochem. J.* **40**, 481.
MANN, T. (1948). *Lancet*, **254**, 446.
MUNRO FOX, H. (1921). *J. Gen. Physiol.* **3**, 501.
ROTHSCHILD, LORD (1947*a*). *Nature, Lond.*, **159**, 134.
ROTHSCHILD, LORD (1947*b*). *J. Exp. Biol.* **24**, 390.
TOSIC, J. & WALTON, A. (1947). *J. Agric. Sci.* **37**, 69.

EXPLANATION OF PLATE

Plate 4*a*, i–v. Undiluted ram semen: variation in frequency of impedance changes with time. The bridge unbalance corresponds to a 0·25% change in the standard arm resistance (0·1 in 50 Ω at 5000 cyc.). The vertical white lines are ½ min. apart in all records. The first vertical white line in each record corresponds to 10·5, 30·5, 51·5, 72·0 and 150 min. respectively from the start of the experiment.

Plate 4*b*. Ram semen diluted 1 : 1 with glucose-free Ringer: variation in frequency of impedance changes with temperature. i, 16·0° C.; ii, 36·0° C.



ROTHSCHILD—THE ACTIVITY OF RAM SPERMATOZOA

VAPOUR PRESSURE CHANGES IN THE FROG'S EGG AT FERTILIZATION

By L. E. R. PICKEN and LORD ROTHSCHILD

From the Department of Zoology, University of Cambridge

(*Received* 4 *February* 1948)

(With Two Text-figures)

INTRODUCTION

The suggestion has been made that the response of an egg to the action of a spermatozoon or parthenogenetic agent may be similar to that of a nerve to stimulation (Lillie, 1909). If this were so, it might be expected that activation, whether by a parthenogenetic agent or by a spermatozoon, would cause an increase in the permeability of the egg surface; although this increase in permeability is transient in the case of stimulated nerve, muscle, or *Nitella* cells, it need not necessarily be so. On the other hand, though the plasma membrane of the sea-urchin egg is known to become more permeable to sparingly ionized substances after fertilization (Lillie, 1917), there is no evidence that it is more permeable to ions, as would be expected if activation were strictly analogous to the stimulation of nerve or muscle. There is, in fact, some evidence to the contrary; the most recent investigations make it doubtful if any decrease in membrane resistance after fertilization has ever been measured in sea-urchin eggs (Cole, 1928).

In the frog's egg, which, like that of the sea-urchin, has been studied intensively, profound changes in osmotic pressure were said by Backmann & Runnström (1912, p. 344) to take place after fertilization: 'Durch die Befruchtung wird in der Eizelle von *Rana temporaria* eine erhebliche Reduktion des osmotischen Druckes des Ei-inhaltes hervorgerufen. Der osmotische Druck wird etwa bis zu einem Zehntel des osmotischen Druckes beim erwachsenen Frosch reduziert. Es herrscht Isotonie zwischen dem eben befruchteten Froschei und dem umgebenden Wasser.'*

Their results are displayed in Table 1. From these it would seem that the fall

Table 1. *Freezing-point depressions* (Δ).

	°C.
R. temporaria serum	0·456
Ripe unfertilized eggs	0·480
Pond water	0·060
Tap water	0·015
Jelly	0·015
Fertilized eggs (soon after fertilization)	0·045
Unfertilized eggs (in tap water)	
Time: 3 hr.	0·350
25 hr.	0·197
36 hr.	0·235
43 hr.	0·575

* 'In *R. temporaria*, a considerable reduction in the osmotic pressure of the egg contents is caused by fertilization. The osmotic pressure is reduced to about a tenth of that of the fully grown frog. Isotonicity prevails between the frog's egg which has just been fertilized and the surrounding water.'

in osmotic pressure in unfertilized eggs in tap water is much less than in fertilized eggs soon after fertilization; even after 36 hr. the fall in osmotic pressure in unfertilized eggs in tap water is not as great as in fertilized eggs.

The interpretation of the results given in the text of Backmann and Runnström's paper, however, seems to differ from that expressed in the summary already quoted. In the text it is suggested that the fall in osmotic pressure is due not to fertilization, but to the hypotonicity of the pond water, which progressively cytolyses the eggs in the absence of the stabilizing or corrective effect of fertilization or parthenogenetic activation.

Further work by other experimenters in this field (Backmann & Sundberg, 1912; Białaszewicz, 1912; Przyłęçki, 1917; Voss, 1926; Krogh, Schmidt-Nielsen & Zeuthen, 1938) has failed to make clear the relation between osmotic pressure changes and fertilization, and it therefore seemed advisable to repeat Backmann & Runnström's experiments, measuring vapour pressures rather than freezing-point depressions, in order to establish what change in vapour pressure occurs in the frog's egg on fertilization, and in the unfertilized egg on transference to tap water.

MATERIAL

The eggs of the common frog, *R. temporaria*, were used. In some experiments measurements were made on unfertilized, 'uterine' eggs, while in others 'coelomic' eggs were used. The latter are eggs which have left the ovaries but have not as yet entered the oviducts. Coelomic eggs are very convenient material since they lack a covering of jelly.

The method of obtaining high percentages of fertilized eggs was as follows. A frog was pithed, and the eggs from the two 'uteri' were spread in a layer one egg thick over the bottom of two Petri dishes, about 10 cm. in diameter. A third Petri dish of the same size was half-filled with tap water and two testes and two seminal vesicles were placed in it. These were then crushed, and the contents of the dish stirred to give a homogeneous suspension of sperm. This was poured over the eggs in one Petri dish, and left for 10 min. Simultaneously, a similar volume of tap water was poured over the control eggs in the other dish. After 10 min. the two dishes were placed separately in large volumes of tap water. In general a high percentage of eggs was fertilized and cleaved normally; no control eggs cleaved. As Cambridge tap water was not at that time (1935) of constant composition, artificial media of standard composition were sometimes used. Cleavage and development were normal in these.

MORPHOLOGY

'Uterine' eggs are covered with a dense layer of jelly, which cannot be dissected off without damaging the eggs. They are also surrounded by a membrane (between egg and jelly), often known as the vitelline membrane, but which, in accordance with the nomenclature accepted for the trout egg, it is here proposed to call the chorion. When the unfertilized frog's egg is placed in tap water, the chorion becomes thicker and tougher, as in the trout egg. After being in water for some time, a space appears between the chorion and cell surface; this is the perivitelline space and is

filled with fluid. When it appears, the egg becomes free to rotate, until its centre of gravity is in the equilibrium position, and sinks to the bottom of the perivitelline space. If an egg with a well-defined white pole is now turned upside down, it rotates until the white pole, towards which the centre of gravity lies, once more faces vertically downwards. Here there is a parallel in behaviour between the frog's egg and the trout egg.*

When an egg is fertilized the same phenomena are observed, but the perivitelline space appears more rapidly than in the unfertilized egg. After eggs have been successfully inseminated, they will rotate in about 20 min.; in the case of unfertilized eggs, a period of $1\frac{1}{2}$–3 hr. may elapse before rotation occurs.

METHOD

The vapour pressure of the contents of fertilized and unfertilized eggs was determined by the differential thermal method of Hill (1930), which has advantages over the Beckmann freezing-point depression technique used by Backmann & Runnström and most other experimenters in this field. The instrument for determining vapour pressures was similar to that described by Hill (1930) and Margaria (1930). Two thermopiles were used for each determination, and their two readings were averaged. A number of determinations were made in an atmosphere of nitrogen to see if the heat production of material obtained by squashing the eggs caused any appreciable error. The results were negative. All manipulations were made in a small moist chamber (Picken, 1936).

RESULTS

As a preliminary experiment, and as a check on the method, the vapour pressure of frog's blood was determined. Ten pairs of determinations were made, and the results, expressed as decimal fractions of the vapour pressure of a 1 % NaCl solution,

Table 2

Frog no.	Vapour pressure of frogs' blood in terms of a 1 % NaCl solution
1	0·62
2	0·66
3	0·72
4	0·65
5	0·84
6	0·64
7	0·72
8	0·68
9	0·62
10	0·56

are shown in Table 2. It is clear that there is considerable variation between one frog and another, and this has to be taken into consideration in all experiments described in this paper. The values for the vapour pressure of the eggs of different females also vary considerably from one female to another, but are closely correlated with the values for the vapour pressure of the blood from the same females (Fig. 1).

* See, however, Rothschild (1947) for a discussion of this phenomenon in the egg of the trout and frog.

Results of measurements on unfertilized 'coelomic' eggs and on ripe 'uterine' eggs are shown in Table 3. In these experiments the material obtained by squashing five eggs, or alternatively by sucking up the contents of five eggs in a fine glass pipette, was placed on a filter-paper applied to one face of a thermopile. A 1% solution of NaCl was applied to the filter-paper on the other face of the thermopile.

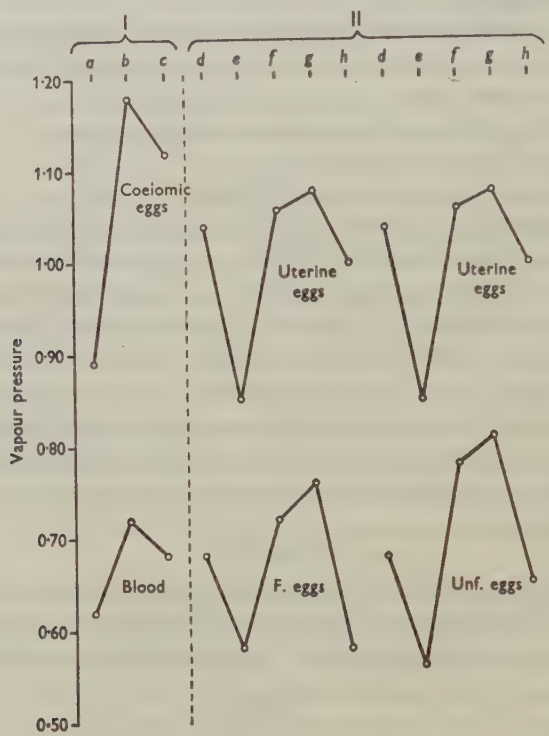


Fig. 1. I, Correlation between vapour pressure of blood and coelomic eggs; II, correlation between vapour pressure of uterine, unfertilized and fertilized eggs. Vapour pressure in terms of 1% NaCl solution. F. eggs = fertilized eggs after 3 hr. in water; Unf. eggs = unfertilized eggs after 3 hr. in water. The letters *a*...*h* refer to different frogs.

Table 3. *Vapour pressures expressed as decimal fractions of the vapour pressure of a 1% NaCl solution*

Frog no.	'Coelomic' eggs	Frog no.	'Uterine' eggs
11	0·89	16	1·00
12	1·18	17	1·04
13	1·12	18	0·85
14	0·92	19	0·88
15	1·18	20	1·00
—	—	21	1·06
—	—	22	1·08
Arithmetic mean	1·06		0·99
Δ*	0·62° C.		0·58° C.

* Calculated equivalent depression of freezing-point.

The main experiments on fertilized and unfertilized eggs were carried out after the eggs had been in water for half an hour, during which time the jelly swells. Five eggs were selected, and the jelly surrounding each was carefully dissected off with micro-scissors, leaving the eggs bare except for the chorion, which was not removed. The eggs were then ground up, and the pulp was placed on a filter-paper on the face of a thermopile.

The experiments fall into two groups: first, measurements of vapour pressure made half an hour after insemination; and secondly, measurements made 3 hr. after insemination. All fertilized eggs cleaved within 3 hr., but no fertilized eggs cleaved within half an hour of insemination; difficulties therefore arose in selecting unquestionably fertilized eggs from the fertilized batch, half an hour after insemination. At the beginning of the season, all ripe eggs have a well-defined animal and vegetative pole. As the fertilized eggs begin to rotate about 20 min. after insemination, it is possible, at the beginning of the season, to select fertilized eggs after half an hour by removing those which have clearly undergone rotation. All eggs, however, do not have a well-defined white pole, particularly when the breeding season is advanced. In these circumstances there is no visible difference between the animal and vegetative pole, and it is impossible to observe rotation, though the eggs cleave in the normal way. It was, therefore, thought inadvisable to select eggs which had rotated and were thus known to be fertilized, when in other experiments selection was necessarily random, since no rotation was visible. Had this selection been made, the results of different experiments (with and without selection) would not have been comparable. In the half-hour experiments, therefore, eggs from the inseminated batches were selected at random, no attention being paid to rotation or lack of rotation. After the selection had been made, the inseminated eggs were left in their Petri dish for 3 hr., by which time the first cleavage had taken place. Each dish held about 600 eggs. The total number of cleaved and uncleaved eggs was then counted. The results of these experiments are shown in Table 4.

Table 4. *Vapour pressure of unfertilized and fertilized frogs' eggs, expressed as decimal fractions of the vapour pressure of a* 1% *NaCl solution. Fertilized eggs,* 30 *min. after insemination; unfertilized eggs,* 30 *min. after immersion in water.*

Frog no.	Unfertilized eggs	Ratio of unfertilized to fertilized eggs in unfertilized batch	Fertilized eggs	Ratio of fertilized to unfertilized eggs in fertilized batch
		Unf. : F.		Unf. : F.
23	0·76	100 : 0	0·88	100 : 6
24	0·79	100 : 0	0·72	100 : 10
25	1·10	100 : 0	0·92	100 : 61
26	0·83	100 : 0	0·80	100 : 54
27	0·83	100 : 0	0·74	100 : 3
28	1·12	100 : 0	0·99	100 : 12
29	1·01	100 : 0	0·87	100 : 3
30	0·95	100 : 0	0·97	100 : 8
31	0·91	100 : 0	0·74	100 : 16
32	0·80	100 : 0	0·86	100 : 140
33	0·88	100 : 0	0·77	100 : 42
Arithmetic mean	0·91		0·84	
Δ	0·54° C.		0·50° C.	

The arithmetic means of these results suggest that the fall in vapour pressure in fertilized eggs is greater than in unfertilized eggs, though in both fertilized and unfertilized eggs in water the vapour pressure is lower than in unfertilized eggs before immersion in tap water. It is difficult to analyse these results statistically, because the eggs from different frogs must be considered as different populations. Individual results, however, show a distinct trend in the same sense as the arithmetic means.

A further series of determinations was made 3 hr. after insemination, that is, while the eggs were in the two-cell stage. At this period Backmann & Runnström obtained very low values for the osmotic pressure. They claimed that soon after fertilization the osmotic pressure of the egg contents was about the same as that of pond water, though the osmotic pressure of the unfertilized eggs after 3 hr. in water was considerably higher. The uncertainty in determinations made half an hour after insemination—that is, whether eggs were fertilized or not—did not arise in these experiments, since only eggs which had cleaved were used. The values recorded are shown in Table 5. One anomalous result was obtained, in which fertilized eggs

Table 5. *Vapour pressures expressed as decimal fractions of a* 1% *NaCl solution*

Frog no.	Unfertilized eggs	Fertilized eggs at 1st cleavage
34	0·81	0·76
35	0·65	0·58
36	—	0·57
37	0·68	0·68
38	0·78	0·72
39	0·56	0·58
40	0·60	0·62
41	0·60	—
Arithmetic mean	0·67	0·64
Δ	0·40° C.	0·38° C.

had a vapour pressure of 0·69 and unfertilized eggs 0·55% NaCl. The difference in vapour pressure between fertilized and unfertilized eggs 3 hr. after insemination and immersion in water is not significant.

To test this result a few experiments were made with material from unfertilized eggs on one thermopile face and that from fertilized eggs on the other. These experiments confirmed the results given in Table 5 and are in serious disagreement with those of Backmann & Runnström. The extent of this disagreement can be seen in Fig. 2, where the values for the osmotic pressure of the eggs are compared graphically.

Some preliminary conductivity measurements were carried out on fertilized and unfertilized egg pulp. These experiments, which presented considerable technical difficulties, were made in collaboration with the late Oliver Gatty. The results suggest that after fertilization there is approximately 10% less electrolyte in the egg than before fertilization, which is in qualitative agreement with the results of McClendon (1915); but the experiments should be repeated before the results can be accepted

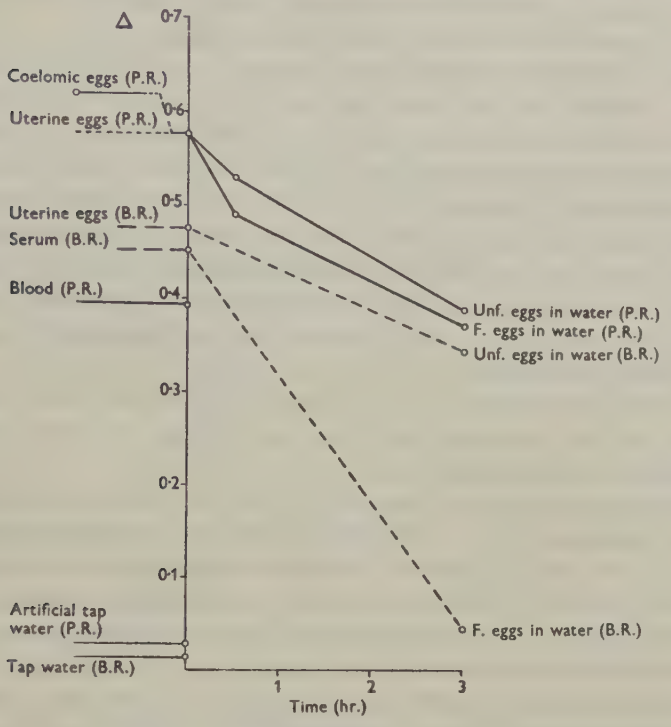


Fig. 2. Comparison of results described in this paper (P.R.) and those of Backmann & Runnström (B.R.).

DISCUSSION

Our experiments show that 3 hr. after fertilization in tap water, or 3 hr. after transfer to tap water, there is no difference between the vapour pressure of fertilized and unfertilized eggs. In both, however, the vapour pressure has fallen to about 0·66 of its initial value. Backmann & Runnström showed that the increase in volume of eggs after fertilization was at most 6·6% and calculated that a water uptake of this amount would lead to a fall in osmotic pressure from that of a 0·7% solution of sodium chloride to that of a 0·58% solution, that is, a fall of about one-sixth. The fall observed by us would therefore be accounted for if the egg were semipermeable and swelled in tap water. This is in fact what Krogh *et al.* (1938), who also used the differential thermal method for measuring vapour pressures, believe to happen in the fertilized egg.

Preliminary conductivity experiments suggested, however, that electrolytes disappear from the egg after fertilization. That is to say, some of the fall in vapour pressure might be due to disappearance of electrolytes from the contents of the egg. The simplest hypothesis to account for this is that outward diffusion of ions into the external medium occurs, until a new dynamic equilibrium between egg and environment is established.

McClendon (1915) obtained similar results, but his experiments might be

criticized on the grounds that the existence of a single dead egg in his suspensions of fertilized eggs would have vitiated his conclusions.

Backmann & Runnström pointed out, however, that fertilized eggs will develop in repeatedly changed distilled water, and argued from this observation that there can be no continuous loss of electrolytes by diffusion from the egg, since the salt content of the egg would soon be exhausted under these conditions, and development would be impossible. They concluded, since the swelling of the fertilized eggs was not sufficient to account for the reduction in osmotic pressure to about one-tenth of the initial value, that the number of osmotically active molecules and ions in the egg substance is reduced as a result of fertilization, not by water entering, nor by ions or molecules diffusing out, but by adsorption within the egg due to a change in the state of the cell colloids.

If, as our results show, there is no such precipitous fall in the vapour pressure of the fertilized egg to isotonicity with the surrounding tap water, Backmann & Runnström's hypothesis is superfluous.

The discrepancy between our results and those of Backmann & Runnström is not easy to understand. The Beckmann method of determining freezing-point depressions is, for various reasons, not entirely satisfactory when dealing with highly viscous material of this kind. About 1 c.c. of fluid is needed for accurate determinations, and this would mean using a large number of eggs for each determination. But in order to remove the jelly completely, 5 or 10 min. may have to be allowed for the dissection of each egg. One source of error may, therefore, have been incomplete removal of the jelly, the presence of which would tend to dilute the egg contents and lead to a low value for the vapour pressure. It is unlikely, however, that errors introduced in this way could account for the discrepancy, which amounts to the difference between the osmotic pressure of frog's blood and that of tap water. Moreover, the results of Backmann & Runnström's determinations on frog's blood and unfertilized eggs are similar to ours, and this again suggests that the discrepancy cannot only be due to the inadequacies of the Beckmann method.

In general agreement with our results, Cole & Guttman (1942) found no significant change in the resistivity of the frog's egg 'cytoplasm' nor in membrane resistance, after fertilization. Though they make no mention of chorion, perivitelline space or conductivity of the perivitelline fluid—all of which might affect the calculations of membrane resistance*—it is hardly conceivable that their alternating current experiments should have failed to reveal a difference between the conductivity of fertilized and unfertilized egg contents, if Backmann & Runnström's results are correct.

The small difference between the vapour pressure of fertilized and unfertilized frog's eggs, half an hour after fertilization, could be accounted for by the more rapid diffusion of salts out of the egg, through the chorion and into the external medium, or of water into the egg, as a result of fertilization. If the salts diffused

* Similar difficulties arise from the presence of the chorion and perivitelline space in the trout egg; but so far, no membrane resistance measurements have been made on this egg (Rothschild, 1946).

out of the egg and into the perivitelline space, but not through the chorion, vapour pressure measurements would only reveal a difference between fertilized and unfertilized eggs if water entered the perivitelline space, since the chorion was not dissected away from the eggs.

As unfertilized eggs develop a perivitelline space after immersion in tap water, the idea that the extrusion of the chorion is a direct result of fertilization must be rejected. It is true that the perivitelline space appears much more quickly in fertilized eggs; but the fact that it can appear, albeit more slowly, without fertilization, after the eggs have been transferred from the 'uterus' to a hypotonic medium, suggests that this transfer is the responsible factor. This was pointed out by Przyłęçki (1917). The spermatozoon appears to alter the rate at which the perivitelline space is formed, or the rate at which a new dynamic equilibrium between the egg and its environment is established. This is confirmed by the experiments of Voss (1926).

The appearance of a perivitelline space in unfertilized eggs in tap water suggests that the hypotonicity of this medium acts as an abortive parthenogenetic stimulus. The spermatozoon seems to be more efficient than the hypotonic medium in causing the perivitelline space to appear; at the same time, perhaps as a result of a change in the cell surface, there is slightly less tendency for osmotic equilibrium to be approached between the egg and its environment than in the fertilized egg.

Thus far there has been no discussion of the mechanism by which the perivitelline space is produced. Its formation may be an osmotic phenomenon due to the liberation of substances of high molecular weight into the space between the chorion and the vitelline membrane (Białaszewicz, 1912); the elevation of the fertilization membrane in sea-urchin eggs is probably due to a reaction of this type (Loeb, 1908). In unfertilized eggs we may suppose that these substances diffuse out more slowly.

Unfertilized frog's eggs swell more than do fertilized eggs in tap water, and in them the fall in vapour pressure seems to be the first step in cytolysis. They do not survive for any length of time in tap water, unlike unfertilized trout eggs, which can be kept in a healthy condition for weeks in this medium. This difference is probably due to the impermeability of the vitelline membrane of the trout egg.

If the analysis of the vapour pressure changes is to be carried further, we require simultaneous measurements of volume changes, vapour pressure changes in the eggs, and electrolyte concentration changes in the eggs and in the surrounding medium. At the moment it seems reasonable to conclude that, if Backmann & Runnström's observations on volume changes are correct, these would more than account for the fall in vapour pressure observed in fertilized and unfertilized eggs, supposing that the increase in volume is due to the uptake of water.

Concerning the postulated change in permeability of the egg surface on fertilization, the vapour pressure experiments provide neither evidence for nor against such a change. They show only that the fall in vapour pressure is of the same order as that which might occur as a result of the volume change. The fact that the contents of the egg do not become isotonic with the surrounding tap water indicates that at

some stage after the egg, whether fertilized or unfertilized, is transferred to tap water, the exchange of water and dissolved substances between egg and surrounding medium is actively controlled.

SUMMARY

1. The vapour pressure of unfertilized and fertilized frogs' eggs has been measured by the differential thermal method.

2. The following results were obtained; they are expressed as decimal fractions of the vapour pressure of a 1% aqueous solution of sodium chloride:

Coelomic eggs (eggs free in the body cavity, before entering the oviducts)	1·06
Uterine eggs (eggs in the distal end of the oviducts)	0·99
Unfertilized eggs, 30 min. after immersion in tap water	0·91
Inseminated eggs, 30 min. after immersion in tap water	0·84
Unfertilized eggs, 180 min. after immersion in tap water	0·67
Fertilized eggs, 180 min. after immersion in tap water	0·64

3. These results are in serious disagreement with those of Backmann & Runnström (1912) and Backmann & Sundberg (1912), but in general agreement with those of Krogh *et al.* (1938).

4. It is concluded that while there is probably a slight difference between the vapour pressure of fertilized eggs in tap water half an hour after fertilization, and of unfertilized eggs half an hour after transfer to tap water, the difference is negligible after 3 hr. The initial difference may be due to a more rapid interchange between egg and environment in fertilized eggs.

5. In the case of unfertilized eggs, the effect of transfer to a hypotonic medium may be interpreted as an abortive response to a parthenogenetic stimulus.

REFERENCES

BACKMANN, E. L. & RUNNSTRÖM, J. (1912). *Pflüg. Arch. ges. Physiol.* **144**, 287.
BACKMANN, E. L. & SUNDBERG, C. (1912). *Pflüg. Arch. ges. Physiol.* **146**, 212.
BIAŁASZEWICZ, K. (1912). *Bull. int. Acad. Cracovie*, B, **1**.
COLE, K. S. (1928). *J. Gen. Physiol.* **12**, 37.
COLE, K. S. & GUTTMAN, R. M. (1942). *J. Gen. Physiol.* **25**, 765.
HILL, A. V. (1930). *Proc. Roy. Soc.* A, **127**, 9.
KROGH, A., SCHMIDT-NIELSEN, K. & ZEUTHEN, E. (1938). *Z. vergl. Physiol.* **26**, 230.
LILLIE, R. S. (1909). *Biol. Bull. Woods Hole*, **17**, 188.
LILLIE, R. S. (1917). *Amer. J. Physiol.* **43**, 43.
LOEB, J. (1908). *Arch EntwMech. Org.* **26**, 82.
MCCLENDON, J. F. (1915). *Amer. J. Physiol.* **38**, 163.
MARGARIA, R. (1930). *J. Physiol.* **70**, 417.
PICKEN, L. E. R. (1936). *J. Exp. Biol.* **13**, 309.
PRZYŁĘCKI, ST J. (1917). *C.R. Soc. Sci. Varsovie.* **3**, 323.
ROTHSCHILD, LORD (1946). *J. Exp. Biol.* **23**, 77.
ROTHSCHILD, LORD (1947). *J. Exp. Biol.* **24**, 390.
VOSS, H. (1926). *Roux Arch. Entw. Mech. Organ.* **107**, 241.

THE PROPULSIVE POWERS OF BLUE AND FIN WHALES

By K. A. KERMACK, *University College, London*

(*Received* 19 *December* 1947)

Gray (1936), discussing the swimming of the dolphin and porpoise, demonstrates that, for the horse-power developed per lb. of muscle to agree with that expected by analogy with man and the dog, the flow past the body of the animal must be free from turbulence. An attempt is made here to repeat his calculations for Blue and Fin whales (*Balaenoptera musculus* and *B. physalus*). This arose out of observations made on these whales by the writer during a recent visit to the Antarctic on the floating factory *Empire Victory*.

The maximum speed of swimming of which these whales are capable can be estimated with considerable accuracy. The whales are hunted by small, fast steam-vessels, known as 'catchers', and one of the favoured methods is for the catcher to come up to the whale and scare it. The whale then makes off at full speed on a straight course, the catcher following full speed ahead. A 14- or 15-knot boat will by this means catch a whale in about an hour. Thus, it may be fairly assumed that the maximum sustained speed, of which one of these whales is capable over this period of time, is approximately 15 knots. Conversations with a number of the whalers tended to confirm this; and also suggested that it was unlikely that the whale, even in its initial burst of speed when chased or harpooned, ever much exceeded 20 knots.*

Table 1

	Length of whale (ft.)	Sex	Weight of muscle†
	Dorsal muscle-mass		
Fin whales	66	♀	5,732
	67	♀	8,245
	69	♀	8,928
	69	♀	8,642
	71	♂	8,068
Blue whale	84	♀	16,931
	Ventral muscle-mass		
Fin whales	66	♀	2,866
	68	♂	3,891
	69	♀	3,968
Blue whale	86	♀	8,377

† Weights are of the muscle on one side only, and are in pounds.

Figures for the weight of locomotory muscle were obtained from some weighings on the Fl. F. *Empire Venture*. These are given above (Table 1). From these figures it may be assumed that a Fin whale 66 ft. in length would have some 17,000 lb. of

* A paper which came to hand after the present work had gone to the printer, McCarthy (1946), tends to confirm these findings. By the use of asdic the Blue whale is estimated to have a maximum speed of 20 knots, and the Fin whale a maximum speed of 14 knots. These speeds were maintained for little more than 10 minutes, then being reduced.

locomotory muscle, and one of 69 ft. 25,000 lb.; while a Blue whale of 85 ft. in length would have some 50,000 lb. of this muscle.

The surface area of the whale was estimated by assuming it to be approximated by two right circular cones with a common base at the transverse section of maximum depth, the diameter of the base being taken as equal to this depth. This approximation is similar to that used by Laurie (1933). A further adjustment was made for the area of the fins.

This method could be checked to some extent by comparing the value of the surface area of a Fin whale obtained in this way with that obtained by scaling up from actual measurements made on a 10 ft. Fin whale foetus, a foetus of this size approximating rather closely in shape to an adult whale. The two values so obtained agreed almost suspiciously well (to closer than 1%), and so the results given by the method of cones, for want of better, have been used in the following calculations.

From these facts the drag of the whale may be calculated from the formula:

$$\text{drag } (D) = \frac{C_f \rho A V^2}{2},$$

where C_f = drag coefficient, ρ = density of the water in which the whale is swimming, A = the whale's surface area and V = its speed of swimming, these units being consistent.

The value of the drag coefficient must be assumed for any given example, and has been taken as being 10% above that for an equivalent flat plate. This assumption may be made since the drag of a streamline body is 10–15% above that of an equivalent flat plate. The drag coefficient will vary with the degree of turbulence in the flow of water over the body of the animal. Values of this coefficient have been calculated for the limiting cases of completely turbulent and laminar flow.

In the first case the formula of Prandtl (1932)

$$C_f = 0{\cdot}0455\ (\log_{10} R)^{-2{\cdot}58}$$

for a flat plate was used, and in the second the formula of Blasius (1908)

$$C_f = 1{\cdot}32824\ R^{-0{\cdot}5}.$$

R in these two formulae is the Reynold's number VL/ν, V being the velocity, L the length of the body, and ν the kinematic viscosity of the surrounding fluid.

The results, calculated for 66 ft. and 69 ft. Fin whales, and for an 85 ft. Blue whale, at speeds of swimming of 15, 20 and 25 knots, are given in Table 2. This table gives in addition the total effective horse-power required, and the effective horse-power output per lb. of locomotory muscle in each case. The propulsive efficiency has been assumed to be 100% throughout, to facilitate direct comparison with Gray (1936).

The values for turbulent flow may be compared with those of Barcroft (1934) and Krogh (1934), when a little calculation will show the present results to be intermediate in value.

Gray (1936), postulating laminar flow, estimated the power output of the dolphin and porpoise to be of the order of 0·01 horse-power per lb. of locomotory muscle. From Table 2 it is clear that even if the flow be completely turbulent, such a specific power output is quite adequate for propulsion in Blue and Fin whales. The

important thing here is, as Gray (1947) has pointed out, the weight of muscle per sq.ft. of surface area. Table 3, due to Gray, shows that in these larger species the muscle weight per sq.ft. of surface area is between six and twenty times as much as in the dolphin and porpoise. This is partly due to the fact that these whales have, relative to their size, rather more muscle than the smaller species; but it is mainly a simple scale effect, the surface area of similar animals varying as the square, and the mass of their muscles as the cube, of their length.

Table 2

Species of whale	Length of whale (ft.)	Surface area in sq.ft.	Speed of swimming in knots	Reynold's number	Assumed nature of flow	Calculated drag coefficient	Drag in lb. wt.	Total h.p.	H.p. per lb. of locomotory muscle
Fin	66	1,239	15	$8{\cdot}825 \times 10^7$	Laminar	0·00016	120·5	5·5	0·00032
					Turbulent	0·0024	1846	83·9	0·00494
			20	$1{\cdot}166 \times 10^8$	Laminar	0·00014	182·7	10·0	0·00064
					Turbulent	0·0023	3113	187	0·01099
			25	$1{\cdot}466 \times 10^8$	Laminar	0·00012	257·8	19·5	0·0011
					Turbulent	0·0023	4795	362	0·0213
	69	1,357	15	$9{\cdot}22 \times 10^7$	Laminar	0·00015	128·9	5·9	0·00023
					Turbulent	0·0024	2021	91·9	0·00367
			20	$1{\cdot}218 \times 10^8$	Laminar	0·00013	195·7	11·7	0·00047
					Turbulent	0·0023	3394	204	0·00814
			25	$1{\cdot}532 \times 10^8$	Laminar	0·00012	276·0	20·8	0·00083
					Turbulent	0·0022	5182	391	0·01564
Blue	85	1,890	15	$1{\cdot}14 \times 10^8$	Laminar	0·00014	161·7	7·4	0·00015
					Turbulent	0·0023	2725	124	0·00248
			20	$1{\cdot}50 \times 10^8$	Laminar	0·00012	245·5	14·7	0·0003
					Turbulent	0·0022	4596	276	0·0055
			25	$1{\cdot}89 \times 10^8$	Laminar	0·00011	346·7	26·2	0·0005
					Turbulent	0·0022	7087	535	0·0107

Table 3

Species	Length (ft.)	Surface area (sq.ft.)	Weight of locomotory muscle (lb.)	Weight of muscle per sq.ft. area
Porpoise	4	7	9	1·3
Dolphin	6	15	35	2·3
Fin whale (1)	66	1,239	17,000	13·7
Fin whale (2)	69	1,357	25,000	18·4
Blue whale	85	1,890	50,000	26·4

Thus, if the same specific power output be assumed for the muscles of the large species as for those of the small, there is no need to postulate laminar flow in the large whales; although, as Gray (1936) has shown, such a postulate is necessary for the dolphin and porpoise.

A turbulent boundary layer is rather to be expected in these large whales, reaching as they do considerably higher Reynold's numbers (10^8 as against 10^7)

than the dolphin; the tendency of a boundary layer to break down into turbulence increasing with increase in the Reynold's number.

The alternative hypothesis, that the specific power output of the muscles is considerably less in the larger than in the smaller Cetacea, although less likely, should still be kept in mind.

SUMMARY

1. If the flow of water over the body of a Blue or a Fin whale be free from turbulence, the horse-power required per lb. of locomotory muscle is of the order of a tenth of the value estimated by Gray (1936) for the porpoise and dolphin.

2. If the flow be turbulent in these large whales the horse-power required per lb. of muscle agrees closely with Gray's estimate for the smaller Cetacea, or for the other types of mammalian muscle that have been investigated.

In conclusion I should like to thank Prof. D. M. S. Watson and Prof. J. B. S. Haldane for their interest in this work; and Prof. J. Gray for his helpful suggestions. I should also like to express my appreciation of much valuable help received from Miss D. M. Carr.

My thanks are also due to the Discovery Committee for permission to make use of a considerable amount of the data.

REFERENCES

BARCROFT, J. (1934). *Features in the Architecture of Physiological Function*, pp. 121–4. Cambridge.
BLASIUS, H. (1908). *Z. Math. Phys.* **56**, 4.
GRAY, J. (1936). *J. Exp. Biol.* **13**, 192.
GRAY, J. (1947). Private communication.
KROGH, A. (1934). *Nature, Lond.*, **133**, 635.
LAURIE, A. (1933). *Discovery Rep.* **7**, 363.
MCCARTHY, W. J. (1946). Report of H.M. Underwater Detection Establishment, ACSIL/ADM/47/173.
PRANDTL, L. (1932). *Göttinger Ergebn.* **4**, 27.

Since going to press a paper on some aspects of the locomotion of whales appeared in *Nature* (Gawn, R. W. L., *Nature, Lond.*, **161**, 44) and should be consulted along with the ensuing correspondence.

STUDIES ON STERILITY AND PRENATAL MORTALITY IN WILD RABBITS

PART IV. THE LOSS OF EMBRYOS AFTER IMPLANTATION

By F. W. ROGERS BRAMBELL and IVOR H. MILLS

From the Department of Zoology, University College of North Wales, Bangor

(*Received* 4 *March* 1948)

(With Seven Text-figures)

INTRODUCTION

The material on which this study is based and the technique employed have been described in a previous paper (Allen, Brambell & Mills, 1947). The experimental error in estimates of prenatal mortality, based on counts of corpora lutea and of implantation sites, was investigated in that paper, as was also the amount and effect of polyovuly and of transperitoneal migration of ova. Since estimates of prenatal mortality occurring after implantation, with which this paper is concerned, are based on comparison of the number of implantation sites and of surviving embryos, they are unaffected by errors in counts of corpora lutea, by the production of two viable ova from a single follicle and by transperitoneal migration of ova, but they are affected by errors in counts of implantation sites. It was pointed out, in the paper referred to, that although there is little likelihood of failure to detect implantation sites containing living embryos the possibility remains of the disappearance before full term of sites in which the embryos had died and were reabsorbed soon after implantation or, more probably, that such sites might be overlooked, through becoming less conspicuous, and hence omitted from the counts. Actually, significant changes were observed in the mean number of implantation sites in the uteri, counted at successive stages of pregnancy. In particular, a decline occurred in the mean number towards the end of gestation, such as would result from the disappearance of approximately 9% of the implantation sites. Although the disappearance of sites would appear to be the simplest explanation of this phenomenon it was by no means clear that it was the correct one, and there were grounds for thinking that it might be more complex. Subsequent experimental investigation of this problem (Brambell, Henderson & Mills, 1948) has shown that sites in which the embryos had been killed at 16 days post-coitum by surgical means persist until full term in pregnant uteri and can be identified as such at the time of parturition. They certainly are less obvious when the uterus is completely distended with large foetuses and might then be easily missed from the counts, although they have not disappeared. The significant decline in the mean number of implantation sites towards the end of gestation may be due to an experimental error arising from

disappearance of sites, or failures to count them, but there may be some other explanation.

It has been shown that the prenatal mortality suffered after implantation is distributed independently of that occurring before implantation (Allen *et al.* 1947). The loss before implantation has been analysed in another paper (Brambell & Mills, 1947*b*). There is, however, a very important and, indeed, fundamental difference between the methods of estimating the two. The loss before implantation in litters that survive is estimated from the number of corpora lutea in the ovaries and of implantation sites in the uteri; that is from animals in which implantation has already taken place, and in which therefore preimplantation loss has terminated. It follows that whereas the loss of whole litters before implantation cannot be detected by this means, since such animals drop out of the sample of pregnancies before implantation sites are formed, the total loss of ova before implantation in litters that survive is displayed. Theoretically, a comparable estimate of post-implantation loss could be obtained from the numbers of new-born young and of placental sites in post-partum animals, but this is impracticable in dealing with a population of wild mammals. Instead the estimate must be based on the sample of pregnant animals obtained at all stages of gestation between implantation and parturition. It must be based on a sample in process of suffering post-implantation loss and in which, therefore, this loss is incomplete. However, a close estimate of the total loss of embryos up to the time of parturition in litters that survive can be derived from animals approaching full term. No information could be obtained regarding the mortality at the time of parturition.

The fact that the post-implantation loss is estimated from a sample of animals taken during the period in which they were actually in process of suffering that loss has another important consequence. Animals which lose a litter entirely through the death of all the embryos must be excluded from the category of pregnancies as soon as the last surviving embryo has died. Only if all the embryos in each such litter died simultaneously would such animals drop out of the category immediately. Actually the embryos die at intervals, and consequently these animals which are in process of losing their litters remain for a time in the category of pregnancies and display a loss of one or more, but not of all, the embryos. Assuming that the successive embryos die at intervals, then in a large sample of such litters the chances of finding 1, 2, 3, ..., $x-1$, embryos dead, where x = the number of implanted embryos, would be equal, and the mean number of dead embryos per litter affected would tend towards $\frac{1}{2}x$. The proportion of dead embryos in such litters would be 0·5. It has been pointed out previously (Brambell & Mills, 1947*b*) that when the prenatal mortality falls on the litters as units the regression lines for the proportion of embryos lost and for the proportion of litters lost on the size of litter at implantation will coincide. This is true of the final result but not of the distribution of the mortality in a sample of litters including those in process of being lost. If l = the proportion of litters in process of being lost, and E = the proportion of dead embryos, then

$$E = \tfrac{1}{2}l. \qquad \text{(i)}$$

Another difference between pre- and post-implantation loss is to be found in the

speed with which the dead embryos are eliminated. Cleaving ova or early blastulae are so small that they disappear rapidly once they are dead. After implantation the embryos are increasingly substantial structures which persist longer, and the placentae, once established, are still more persistent. It follows that litters in which all the embryos have died and are reabsorbing are identifiable for a considerable period thereafter, and the chances of obtaining them are proportionately greater. It has been shown experimentally (Brambell *et al.* 1948) that when all the embryos are killed by means of stilboestrol at $11\frac{1}{2}$–12 days post-coitum they remain identifiable and their age at death can be determined for 3–$3\frac{1}{2}$ days, and at 16 days post-coitum for 4–$4\frac{1}{2}$ days. The placental remains persist as recognizable reabsorption sites for 9–10 days. The dead embryos persist much longer when some of the embryos in the litter survive and the reabsorption sites then appear to be easily recognizable at full term, as mentioned above. It is important that dead litters in which the age at death of the embryos can be determined should not be included in the category of pregnancies, as was done in the earlier papers (Brambell, 1942, 1944) before the experiments referred to had revealed the implications. A living litter, which is developing, only remains for 24 hr. in any given daily age group, but a dead litter, because it has ceased to develop, remains in the age group to which it belonged when the last embryo died until that embryo has disintegrated too much for its age to be determined, say 4 days later. Thus if 20% of the litters died on the 12th day post-coitum we would expect to get in a large sample equal numbers of living and dead litters containing 12-day embryos.

Once dead, embryos become limp and are distorted by the pressure of the uterus. The brain especially collapses and the cervical flexure is lost. The embryo loses its translucent appearance and becomes opaque and whiter in colour, and the embryonic fluids escape from the membranes, which collapse about the embryo. These characteristics remain for a considerable time after the death of the mother and the consequent death of the other embryos, serving to distinguish those embryos which have died before from those which have died after the mother. Only embryos which had quite unmistakably died before the mother were classed as such, those about which there was any doubt being classed as healthy. There was no difficulty in practice in determining which embryos were dead when surviving embryos were present to compare them with, and very little difficulty even when all the embryos were in the same state. Subsequent experience with embryos killed experimentally has convinced us that our criteria were reliable and conservative. The dead embryo, as autolysis proceeds, becomes fragmented and finally unrecognizable. The limb buds remain recognizable as a rule after the rest of the embryo has disintegrated, and it is possible to determine the age of the embryo from these by the use of the figures in the normal-table of Minot & Taylor (1905). The stage at which it ceased to be possible to determine the age of any of the embryos in a pair of uteri thus formed a natural and definable end-point. The placentae and embryonic membranes are much more persistent than the embryos and their remains, projecting from the mesometrial wall into the uterine lumen, forming a swelling on that part of the uterus, can be recognized and readily distinguished from post-partum or post-

abortion placental sites. Animals containing these, but without fragments of embryos sufficient to determine their age, were classed as late reabsorption stages.

There is no doubt that reabsorption, that is the gradual removal *in situ* of the embryos and placentae, mainly by autolysis, is the usual method of disposal. It occurs when only some of the embryos die at any stage of gestation after implantation and when all the embryos die at early stages up to at least the 15th day. It may occur when all the embryos die even at later stages. Experiments have shown (Brambell *et al.* 1948) that abortion occurs as a rule when the embryonic tissues die after the 19th day. Prior to this, the necessary zone of weakness does not appear to have developed in the placenta, which consequently does not become detached. There is evidence, however, that the placenta may in certain circumstances survive for a time the death of the embryos. Thus, even when the embryos die before the 19th day, the placenta may survive and abortion may follow after an initial period of reabsorption. This was found to occur when the embryos were killed by surgical means at 16 days post-coitum. The possibility of the loss of litters by abortion at later stages in the wild rabbits therefore must be considered.

OBSERVATIONS

A total of 1873 pregnant animals was obtained with surviving embryos between 7 and 32 days of age post-coitum. Of these thirty-nine are excluded from the analysis of mortality because, owing to damage to the uteri during cleaning, either the number of implantation sites or of surviving embryos or both could not be determined. The number in the sample differs from that employed for estimating the loss before implantation (Brambell & Mills, 1947*b*) because Series 9, although available for pre-implantation loss, is not available for post-implantation loss; it was necessary for embryological purposes to preserve some swellings in each uterus unopened and hence the loss could not be determined. A few animals in the other series which had the ovaries damaged, preventing the counting of corpora lutea, which had to be excluded from the data for pre-implantation loss, were available for post-implantation loss. The distribution of the mortality according to the number of embryos that become implanted, as shown by the number of implantation sites, in the 1834 litters available is shown in Table 1. The proportion of litters containing reabsorbing sites and the proportion of embryos reabsorbing are shown to the right of the table. Straight regression lines $y-\bar{y}=b\,(x-\bar{x})$ have been fitted to the data, that for the proportion of embryos lost being

$$Y=0{\cdot}00084x+0{\cdot}0709, \qquad \text{(ii)}$$

where x = the number of implantation sites, and that for the proportion of litters showing loss being

$$Y=0{\cdot}02093x+0{\cdot}0866. \qquad \text{(iii)}$$

These regression lines, together with the observed proportions of litters showing loss and of embryos lost for each size of litter at implantation, are shown in Fig. 1. Testing the significance of these regressions presents some difficulty, because the probability of loss varies with the size of litter at the time of implantation and because the number of examples of each size of litter at implantation vary, thus

Table 1. *All data from implantation to full term (7–32 days)*

No. of sites	No. of embryos reabsorbing							Litters			Embryos		
	0	1	2	3	4	5	6	Total	No. with reabsorbing embryos	Proportion with reabsorbing embryos	Total	No. reabsorbing	Proportion reabsorbing
10	3	—	—	—	—	1	—	4	1	0·250	40	5	0·125
9	18	4	1	2	—	—	—	25	7	0·280	225	12	0·053
8	60	8	5	4	1	3	1	82	22	0·268	656	55	0·084
7	195	33	8	5	5	8	1	255	60	0·235	1785	130	0·073
6	353	40	16	15	7	9	—	440	87	0·198	2640	190	0·072
5	368	48	17	18	19	—	—	470	102	0·217	2350	212	0·090
4	305	25	14	13	—	—	—	357	52	0·146	1428	92	0·064
3	122	21	5	—	—	—	—	148	26	0·176	444	31	0·070
2	33	4	—	—	—	—	—	37	4	0·108	74	4	0·054
1	16	—	—	—	—	—	—	16	—	—	16	—	—
Total	1473	183	66	57	32	21	2	1834	361	0·197	9658	731	0·076

weighting the means. Let x be the size of litter at the time of implantation, Y be the probability of loss as determined from the fitted regression, and y be the observed loss in a count of n specimens. Then the variance* from the mean of a single specimen (the expected value of $n(y-Y)^2$) will be

$$\sigma^2 y \equiv Y(1-Y). \tag{iv}$$

Thus the variance in y for the regression will be

$$\int n\sigma^2 y \Big/ \left(\int n\right)^2, \tag{v}$$

and the variance in b, since $\sigma^2 y$ is dependent on x, will be

$$\int n(x-\bar{x})^2\sigma^2 y \Big/ \left\{\int n(x-\bar{x})^2\right\}^2. \tag{vi}$$

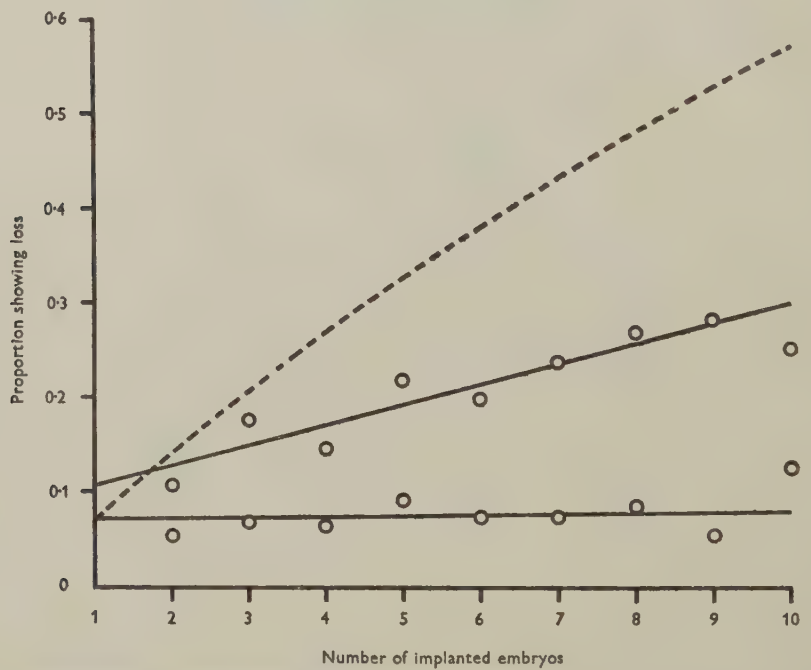


Fig. 1. The proportion of litters showing loss and the proportion of embryos lost according to the size of litter at implantation in the whole sample of pregnancies (7–32 days). The lower circles represent the proportions of embryos lost and the upper circles the proportions of litters showing loss. The solid lines are the fitted regression lines (ii) and (iii) respectively. The broken line represents the expected proportion of litters showing loss (vii) if all the loss fell at random on the embryos as units.

The determination of σy from the values of Y derived from the regression line introduces a small error which can be ignored. Using this method for regression (ii) the probable error of $\bar{y}$ is $\pm 0{\cdot}0027$ and of $b \pm 0{\cdot}0019$, and for regression (iii) the probable error of $\bar{y}$ is $\pm 0{\cdot}0093$ and of $b \pm 0{\cdot}0061$. Hence the proportion of embryos lost does not vary significantly with the size of litter at implantation, but the propor-

* We are indebted to Prof. T. G. Cowling, F.R.S., for this method of estimating the variance.

tion of litters suffering loss does. As was shown in the previous paper on the loss before implantation the slope of the regression line for the proportion of litters showing loss relative to that for the proportion of embryos lost accords with the assumption that part, at least, of the loss is falling upon the embryos as units and is distributed at random amongst them. If the whole of the loss were distributed in this manner the proportion of litters showing loss, L, could be derived from equation (ii), as explained previously, the expression being

$$L = 1 - (0{\cdot}9291 - 0{\cdot}00084x)^x. \qquad \text{(vii)}$$

The resulting curve is represented by the broken line in Fig. 1. It can be seen that the observed proportion of litters showing loss was very much less for all values of x than the expectation on this assumption. This indicates that a substantial part of the loss after implantation may be falling on the litters as units. Comparison of this figure with the corresponding one for loss before implantation (Brambell & Mills, 1947*b*, fig. 1) provides an interesting contrast, for litters lost as units are necessarily excluded from the data for loss before implantation.

Let it be assumed, therefore, that the mortality is made up in part of a loss falling at random on the litters as units, irrespective of size of litter at implantation, and in part of a loss falling at random on the embryos as units. The distribution of a mortality falling at random on the embryos will be given by the expansion of the binomial

$$n(p+q)^x, \qquad \text{(viii)}$$

where $p+q=1$, p is the proportion of embryos lost, x is the number of implantation sites, and n is the number of examples in the litter-size group. Since litters in which all the embryos have died are excluded, *ipso facto*, from the sample, the expectation will fall short of the observations by np^x, thus introducing a small error. This error is negligible for small values of p and large values of x; moreover, in the data under consideration, when x is small so also is n. The mortality falling at random on the litters as units will be distributed amongst the litters so long as they survive in the manner outlined on p. 242, and the proportion of dead embryos in them will be given by equation (i) irrespective of litter size. If a population is suffering both these kinds of mortality, then theoretically litters in process of dying as groups might be subject to a random loss of individual embryos as well. But an individual embryo dying, owing to factors affecting it only, in a litter in which all the other embryos were dying through a common cause, would be indistinguishable in practice and the possibility can be ignored. Hence, in such a population a proportion, l, of the litters will be in process of being lost by the death in sequence of all the embryos, and a proportion, p, of the embryos in the remaining litters will be lost independently of their litter mates. The proportion of dead embryos, E, resulting from both kinds of mortality in a sample of such a population will tend to be

$$E = \tfrac{1}{2}l + p(1-l), \qquad \text{(ix)}$$

irrespective of litter size. The proportion of litters showing loss, L, will be

$$L = lq^x - q^x + 1, \qquad \text{(x)}$$

and therefore will vary with litter size, x. Since the value of E can be determined

from the data a distribution of this kind can be fitted to the data by determining the value of p, or of l, at which the sum of the squares of the deviations of the observed from the expected values is least. Since litters of one cannot show loss they must be omitted. The distribution of best fit for $E=0{\cdot}0758$ is given by $p=0{\cdot}0164$ and $l=0{\cdot}1228$. The theoretical distribution resulting for the 7–32-day sample was tested by means of χ^2, grouping where necessary to avoid values of m below 5. One degree of freedom is lost by the use of E, the proportion of dead embryos and one degree for each litter-size group by the use of n, the number of examples in it, determined from the data. Hence the table is entered with $\chi^2=20{\cdot}5162$, and $N=22$, P being between 0·5 and 0·7. The theoretical distribution therefore fits the data very well. Another test can be applied by comparing the theoretical proportion of litters showing loss, L, derived from equation (x) with the regression line given by equation (iii). Although the distribution of L is curvilinear it approximates to a straight line over the range of most frequent litter sizes and it is legitimate, therefore, to treat it as a straight line over the range $x=5$ to $x=6$ about $\bar{x}=5{\cdot}27$, the values of the constants approximating to $\alpha=0{\cdot}1960$ and $\beta=0{\cdot}0133$. Comparing these with the constants of the regression line (iii), the differences and their probable errors are $\bar{y}-\alpha=0{\cdot}0008\pm0{\cdot}0093$ and $b-\beta=0{\cdot}0076\pm0{\cdot}0061$. Hence it is clear that L does not differ significantly from the straight regression line determined from the data either in slope or in mean in the vicinity of $\bar{x}$.

The evidence from the distribution of the mortality in litters containing at least some surviving embryos is corroborated by the occurrence of many animals in which all the embryos have died and are in various stages of reabsorption. A total of 164 such reabsorbing litters were obtained, of which 101 contained embryos of which the age could be determined and sixty-three were in late stages of reabsorption. As a rule, when the condition of the embryos permitted accurate determination of the age, it was found that all the embryos had died at approximately the same stage of development, and that the least and most advanced embryos did not differ by more than 24 hr. in the stage of development they had attained. Occasionally, a difference of 36 or 48 hr., or even more, was apparent.

The data of the distribution of these reabsorbing litters according to the size of litter at implantation and according to the stage of development attained by the embryos, when this could be determined, are given in Table 2. Combining these with the 1834 living litters given in Table 1 the total numbers of living and dead litters are given in the second column from the right and the second row from the bottom. The proportions of reabsorbing litters presented as a proportion of all living and dead litters, according to age post-coitum and according to the size of litter at implantation, are given in the right-hand column and the bottom row respectively.

The relation of the proportion of reabsorbing litters to size of litter at implantation was investigated by fitting a regression line and testing its significance. The line is given by the formula

$$Y=0{\cdot}1323-0{\cdot}0096x, \qquad \text{(xi)}$$

where $x=$ the size of litter at implantation. The group means and the fitted line are shown in Fig. 2. Since the representation of the data as a proportion of all living and

Table 2. *Distribution of reabsorbing litters*

Days post-coitum	1	2	3	4	5	6	7	8	9	10	11	Total no. of dead litters	Total no. of living and dead litters	Proportion of dead litters
7	—	—	—	—	—	—	—	—	—	—	—	—	104	—
8	—	—	—	—	—	—	—	—	—	—	—	—	78	—
9	—	—	1	—	—	1	—	—	—	—	—	2	76	0·026
10	—	—	1	—	—	—	—	—	—	—	—	1	65	0·015
11	—	—	2	1	5	2	1	1	—	—	1	13	76	0·171
12	2	1	2	4	7	6	2	4	—	—	—	28	116	0·241
13	—	2	2	1	6	8	2	1	—	—	—	22	88	0·250
14	—	—	1	2	5	5	1	—	—	—	—	14	58	0·241
15	—	—	2	—	4	2	1	—	—	—	—	9	120	0·075
16–20	—	—	1	1	1	1	2	—	—	—	—	6	395	0·015
21–25	—	—	—	2	—	—	1	1	—	—	—	4	307	0·013
26–32	—	—	—	2	—	—	—	—	—	—	—	2	452	0·004
Age not determinable	1	1	11	20	14	10	3	1	2	—	—	63	63	—
Total no. of dead litters	3	4	23	33	42	35	13	8	2	—	1	164	—	—
Total no. of living and dead litters	19	41	171	390	512	475	268	90	27	4	1	—	1998	—
Proportion of dead litters	0·158	0·098	0·135	0·085	0·082	0·074	0·049	0·089	0·074	—	—		—	0·0821

dead litters is arbitrary no significance can be attached to the mean. The problem is whether the proportion of reabsorbing litters declines significantly with increasing size of litter at implantation. Since $b = -0{\cdot}0096 \pm 0{\cdot}004$ it must be judged to be significant, though barely so, since it is slightly more than twice its probable error, calculated from equation (vi) above. It must be concluded that the greater the number of embryos which become implanted the less is the probability of the litter being lost. It was shown in earlier papers (Brambell, 1942, 1944) that the proportion of litters showing loss is closely related to the initial size of litter. Since then the

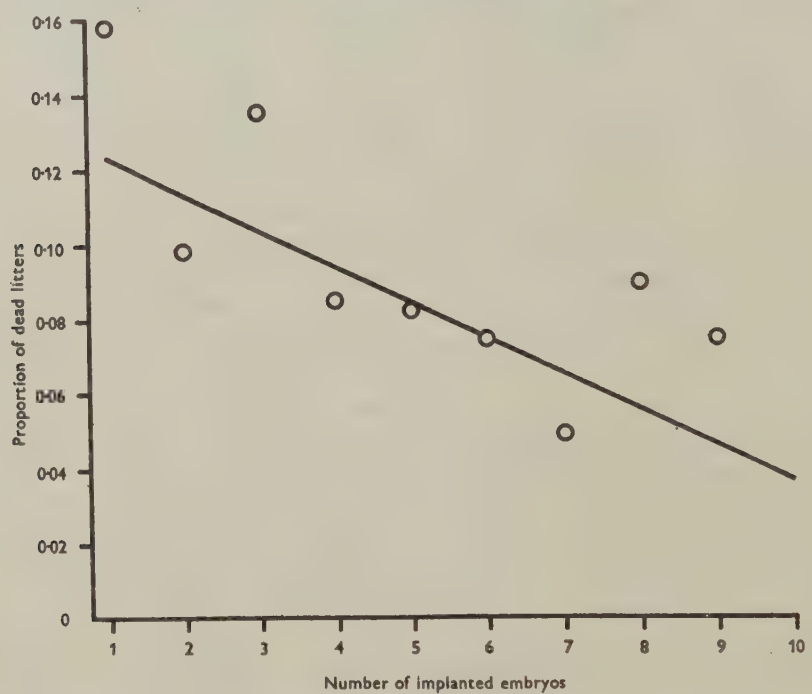


Fig. 2. The proportion of reabsorbing litters with all the embryos dead expressed as a proportion of all litters, surviving and dead. The solid line represents the fitted regression line (xi).

complete separation of the mortality into that suffered before implantation and that after, and the much more extensive material available has enabled us to show that that part of the loss which falls on the embryos as units, and is distributed at random amongst the litters, accounts entirely for this positive relation of the proportion of litters showing loss to initial size of litter. That part of the loss after implantation which falls on the litters as units does not fall with greater severity on the larger litters as compared to the smaller, but rather the reverse. This is a conclusion likely to be of considerable significance in relation to any attempt to determine the cause of the loss of whole litters after implantation.

The daily proportion of litters with all embryos reabsorbing, represented as a proportion of all living and dead litters shown in the right-hand column of Table 2 and in Fig. 3, rises abruptly on the 11th day, reaches a maximum on the 13th and

declines abruptly on the 15th day post-coitum, thereafter remaining at a very low level throughout the remainder of gestation. Since the ages of these dead litters were determined from the least autolysed embryos, the age is the stage at which the last surviving embryo died. It is, therefore, clear that the majority of litters which are reabsorbed *in toto* are lost between the 11th and 15th days inclusive. Very few litters with all the embryos dead and reabsorbing were encountered which had died after the 15th day. It follows that if many litters die after the 15th day they are not reabsorbed but must be aborted. The possibility of the loss of whole litters on the 7th to 10th days has been discussed previously (Brambell & Mills, 1947*a*) and presents a special problem. Many litters of this age were experienced in which all the embryos displayed abnormalities. It is possible that these abnormalities preceded

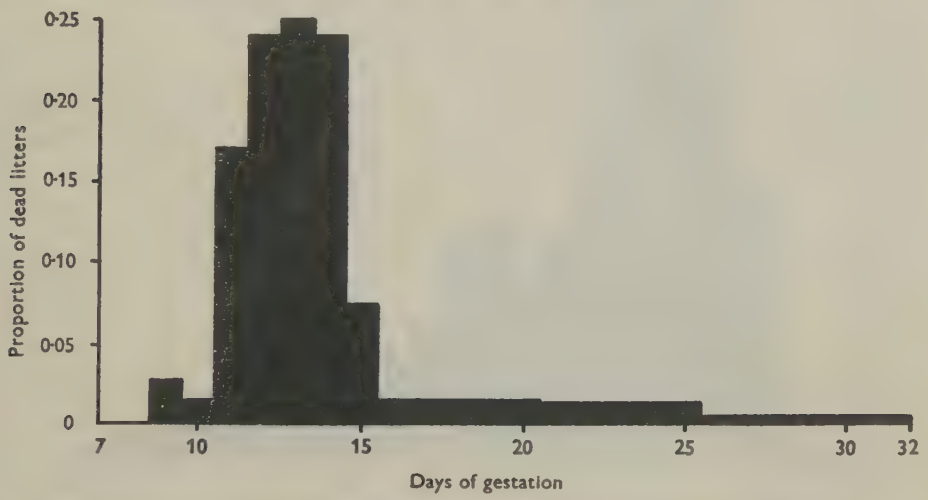


Fig. 3. The proportion of dead litters represented as a proportion of all living and dead litters for each day of gestation.

the death of the embryos and that the affected litters would have continued to develop until on or after the 11th day, and that the group of litters shown in Table 2 as being lost between the 11th and 15th days inclusive represent a later and final stage of these abnormal litters. It is also possible that the abnormalities observed are post-mortem changes in the embryos which had already died between the 7th and 10th days inclusive. If this were the case it might be assumed that the very small embryos would disintegrate so rapidly that it would speedily be impossible to determine their age macroscopically and so they would come mainly into the group of late reabsorption stages. The first possibility appears the more probable since it is very difficult to believe that a further heavy loss of whole litters occurs before the 11th day, not shown in Table 2, which the second possibility involves.

Since it is clear that the majority of litters which are reabsorbed die between the 11th and 15th days inclusive, this age group of living litters should include most of those in process of being lost but in which at least one embryo still survives. The proportion of living litters containing reabsorbing embryos for each day of gestation

from the time of implantation is represented graphically in Fig. 4. It can be seen that the proportion rises sharply on the 9th, 10th and 11th days to a maximum on the 12th. The rise is thus 1 day in advance of the rise in the proportion of dead litters, as shown in Fig. 3. There is no appreciable decline in the proportion of litters containing reabsorbing embryos until the 15th day, when it is sharp, but thereafter the decline is more gradual until the 23rd day when only 4% of litters show loss and, apart from minor fluctuations, this level is maintained thereafter. The peak at 26 days cannot be regarded as significant since it is isolated and depends on too few animals. Comparison with Fig. 3 shows that the considerable proportion

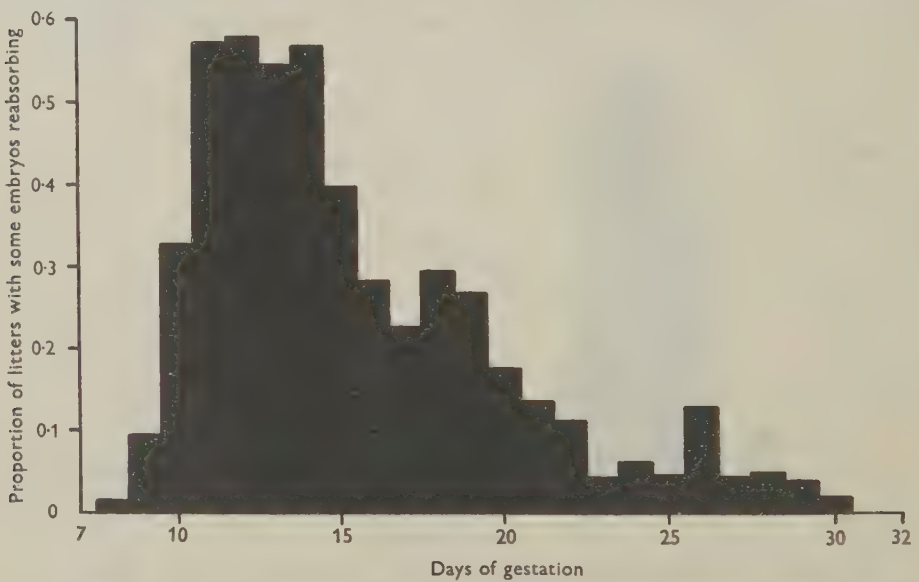


Fig. 4. The proportion of litters with some, but not all, embryos reabsorbing represented as a proportion of all living litters for each day of gestation.

of living litters with some embryos reabsorbing experienced between the 16th and 22nd days inclusive is not accompanied by a corresponding proportion of litters with all embryos reabsorbing. Therefore, these litters cannot all be accounted for by assuming that all the embryos die and are reabsorbed. Yet the apparent loss declines subsequently, as gestation proceeds, until, at the end of gestation only approximately 5% of the surviving litters appear to have suffered loss. There are three possible explanations: either those litters in excess of 5% showing loss in the 16–22-day period survive but the reabsorption sites in them disappear, or they are lost by being aborted, not reabsorbed, or both. It has been shown previously (Allen *et al.* 1947) that the mean number of implantation sites declines significantly as gestation proceeds, from a maximum of $5{\cdot}419 \pm 0{\cdot}070$ in the 16–20-day age group to a minimum of $4{\cdot}923 \pm 0{\cdot}067$ in the 26–32-day age group. This is difficult to understand otherwise than by supposing that some of the sites, presumably those of which the embryos have died and have been reabsorbed relatively soon after

implantation, have either actually disappeared or have become so indistinct as to be frequently overlooked in the counts towards the end of gestation. It is more probable that they are overlooked in the counts than that they disappear entirely for it has been shown (Brambell *et al.* 1948) that when some of the embryos are killed experimentally at 16 days of age the reabsorption sites remain clearly recognizable until full term. It has been shown by Reynolds (1946) that in the rabbit between the 20 and 24 days of gestation, owing to rotation of the embryos, the previously separate and distinct swellings on the uterus merge, the whole uterus becoming equally distended and cylindrical. Inevitably it is much easier to overlook a small reabsorption site, lying between two healthy placentae, in a uterus that has attained this evenly distended cylindrical stage, than at earlier stages when the

Table 3. *Daily mean number of dead embryos per litter suffering loss after implantation*

Days post-coitum	No. of litters with re-absorbing embryos	No. of implantation sites	No. of embryos reabsorbing	Mean no. of embryos re-absorbing per litter	Proportion of embryos reabsorbing
7	0	0	0	—	—
8	1	7	1	—	—
9	7	46	17	2·43	0·37
10	21	131	41	1·95	0·31
11	36	174	87	2·42	0·50
12	51	299	139	2·73	0·46
13	36	195	92	2·56	0·47
14	25	140	60	2·40	0·43
15	44	245	97	2·20	0·40
16	27	148	47	1·74	0·32
17	18	106	21	1·17	0·20
18	12	66	17	1·42	0·26
19	8	43	13	1·63	0·30
20	25	133	37	1·48	0·28
21–32	50	262	62	1·24	0·24

uterus is constricted between successive placentae, whether healthy or reabsorbing. It appears, therefore, quite probable that some early reabsorption sites might be overlooked, even if they had not disappeared, from counts made after the 20th day of gestation. Abortion is not inherently improbable either, since it was found (Brambell *et al.* 1948) that it is the usual sequel to the death of the embryos after the 16th day, when experimentally induced. It should be remembered that, whereas only a dead embryo can be reabsorbed, living embryos as well as dead can be aborted and hence it is possible for a litter to be lost in this way at a time when some of the embryos were still surviving. As has been indicated above, if all the embryos died before any were removed, as is the case when the whole litter is reabsorbed, the mean number of dead embryos per litter in process of being lost would approximate to $\frac{1}{2}x$, where x is the number of implanted embryos. The mean number of dead embryos in litters in process of being lost by abortion would be less if abortion occurred while any of the embryos were still living. The daily mean numbers of dead embryos per litter suffering loss after implantation given in Table 3 are relevant in this connexion. It is apparent that 0·5 of the embryos in litters suffering loss are dead

on the 11th day, so that the value of $\frac{1}{2}x$ is actually attained then, and that the proportion remains high for the succeeding 4 days. The subsequent sharp fall is consistent with either a random loss of embryos in litters that survive or with loss of litters by abortion before all the embryos have died, or both, but not with reabsorption of a large proportion of the litters. It should be borne in mind that the proportion of $\frac{1}{2}x$ dead embryos in litters in process of being lost by reabsorption will be attained only when the proportions of litters entering and leaving this category are equal. Thus, if more litters are entering on the process of dying than are finishing it the value will be less, and vice versa. Hence, if reabsorption of litters were the dominant factor the proportion of dead embryos per litter suffering loss should rise, not fall, after the peak of the mortality has been passed.

Analysis of the mortality in the 7–32-day age group is complicated by the fact that the loss of whole litters does not proceed at a uniform intensity throughout this period, and that, therefore, it is based on animals killed before, during, and after the period of maximum loss. It is necessary, therefore, to fractionate the data, and to divide them up into as many successive shorter age groups as the numbers warrant. The data have been divided into five age groups, 7–10, 11–15, 16–20, 21–25 and 26–32 days respectively, each of which contains data of upwards of 300 animals, and which have also been selected so that the whole of the period of maximum loss of litters, 11–15 days, is concentrated in one age group. The distributions of the data for these age groups are summarized in Table 4. Theoretical distributions of the kind given by equation (ix) have been fitted to each, in a manner similar to that for the 7–32-day age group, and tested by means of χ^2. The expected and theoretical values agree well except in the case of the 21–25-day age group, where the value of P is just below 0·1. The values of p and of l for each age group are given in Table 5, together with the resulting expectations of number of litters showing loss and of number of dead embryos for samples of the given sizes, and the observed numbers for comparison. Mean values of $p = 0{\cdot}0175$ and $l = 0{\cdot}1225$, for the whole sample can be calculated simply from the values for each age group and the number of litters in it. These values approximate sufficiently closely to the values $p = 0{\cdot}0164$ and $l = 0{\cdot}1228$ calculated directly from the 7–32-day age group as a whole. The values of p for the successive age groups rise initially to a maximum for the 16–20-day age group and thereafter decline. The initial rise is to be expected and should continue throughout the period during which the random loss of embryos as units is occurring. The subsequent decline can be accounted for only by a disappearance of reabsorption sites in surviving litters, or by the disappearance of many of those litters which have suffered loss, presumably by abortion since sufficient reabsorptions have not been observed to account for it. That it is due to the disappearance from the counts of reabsorption sites appears the more probable, and the decline in the mean number of implantation sites observed in these age groups, previously recorded (Allen *et al.* 1947) is adequate to account for it. Thus it would appear that the real loss of embryos in litters that survive to full term is considerably in excess of the apparent loss, as shown by the 26–32-day age group, and is not less than 3·5%. A further loss may occur after the 20th day and be

Table 4. *All data from implantation to full term according to age group*

Age group (days)	No. of embryos reabsorbing							Litters			Embryos		
	0	1	2	3	4	5	6	Total	No. with reabsorbing embryos	Proportion with reabsorbing embryos	Total	No. reabsorbing	Proportion reabsorbing
7–10	291	13	8	4	2	2	—	320	29	0·091	1723	59	0·034
11–15	180	65	38	43	27	17	2	372	192	0·516	1974	475	0·241
16–20	299	63	14	9	3	1	—	389	90	0·231	2128	135	0·063
21–25	276	22	3	1	—	1	—	303	27	0·089	1598	36	0·022
26–32	427	20	3	—	—	—	—	450	23	0·051	2235	26	0·012
Total	1473	183	66	57	32	21	2	1834	361	0·197	9658	731	0·076

Table 5. *Observed and expected loss in each age group*

Age group (days)	Observed			Expected						Reabsorbing litters
	Total no. of litters exceeding one	No. of embryos lost	No. of litters showing loss	p	No. of embryos lost	No. of litters showing loss	l	No. of embryos lost	No. of litters showing loss	
7–10	315	59	29	0·0050	8·1	8·0	0·0592	50·9	18·6	3
11–15	370	475	192	0·0250	26·6	25·3	0·4545	448·1	168·1	86
16–20	388	135	90	0·0350	69·8	64·2	0·0613	65·2	23·8	6
21–25	302	36	27	0·0128	20·0	19·4	0·0200	16·0	6·0	4
26–32	443	26	23	0·0070	15·2	15·1	0·0094	10·5	4·2	2

masked by the concurrent disappearance of earlier reabsorption sites proceeding at a faster rate.

The proportion of litters in process of being lost reaches a maximum of 0·4545 in the 11–15-day age group and declines thereafter to 0·0094 in the latest age group. The numbers of litters in process of being lost represented by the values of l for samples of the given sizes are given in the second column from the right of Table 5 and, in the right-hand column, the numbers of dead and reabsorbing litters observed for comparison. It will be seen that, whereas in the 11–15-day period there are approximately twice as many litters dying as dead, in the 16–20-day period, there are almost four times as many. This suggests that some of the litters dying after the 15th day may be lost by abortion, a suggestion which is supported by the fall in the proportion of dead embryos per litter suffering loss, shown in Table 3 and referred to on pp. 253–4, but the numbers of litters involved scarcely warrant a definite conclusion.

Table 6. $E=\frac{1}{2}l+p(1-l)$

Day	E	p	l
9	0·0412	0·005	0·0731
10	0·1114	0·005	0·2149
11	0·2736	0·025	0·5234
12	0·2908	0·025	0·5595
13	0·2667	0·025	0·5088
14	0·2667	0·025	0·5088
15	0·1601	0·025	0·2844
16	0·0916	0·035	0·1217
17	0·0472	0·035	0·0262
18	0·0756	0·035	0·0873
19	0·0788	0·035	0·0942
20	0·0475	0·035	0·0269

The problem of estimating the actual proportion of litters that are lost entirely presents great difficulty, as has been indicated previously (Brambell, 1942, 1944). Several methods have been employed, but all are open to objection for one reason or another. The obvious method of estimating the proportion of litters lost after implantation is from the proportion of living litters that are in process of dying. This is complicated by not knowing how long a litter takes to die, that is, the period elapsing between the deaths of the first and last embryos. Consequently, we do not know how long these litters remain in the sample of pregnancies nor at what rate they are dropping out of the mortality tables. Clearly, in the absence of this knowledge, the best estimate available is provided by the maximum proportion of litters in process of being lost. Since the major part of the loss occurs during so short a time, the estimate for the 11–15-day age group covers too long a period for the purpose, yet the size of the sample does not warrant dividing it further for the purpose of fitting a theoretical distribution. However, the proportion of embryos lost in litters that survive, p, varies comparatively little and justifies the assumption that it is approximately constant for each day within each age group. Then it is possible to estimate l, the proportion of litters in process of being lost, for each day from the values of p in Table 5 and of E, corrected by omitting litters of one. The results are given in Table 6. It will be seen that the proportion of litters in process

of being lost exceeds 0·50 on the 11–14 days inclusive and reaches a maximum of 0·56 on the 12th day. The total proportion of litters lost after implantation should exceed the maximum by the proportion lost before the 12th day plus the proportion which begin to die after the 12th day. It must be concluded, from this method of estimation, that not less than 56% of the litters are lost after implantation, unless the development of the still-surviving embryos in the litters in process of being lost is actually retarded, relative to the embryos in the healthy litters, so that they remain at a given stage of development for longer than normal. This would have the effect of raising the apparent proportion of litters in process of being lost and so increasing the estimate.

Another method of estimating the proportion of litters lost is from the proportion of living and dead litters obtained. Living litters go on developing and consequently remain of any given number of days of age for 24 hr. Dead litters, having ceased to develop, remain at the same stage of development for as long as the age of the embryos is determinable; thus, if the dead embryos remain in a state where the age is determinable for 3 days, the chances of obtaining them are three times as great as if they remained in that state for only 1 day. The mean number of litters obtained per day of gestation from 0 to 8 days post-coitum inclusive, excluding for this purpose those not available for the mortality tables because of damage to the uterus, was 93·3. This is calculated for the period prior to the 9th day, when the loss of whole litters begins to occur and provides an estimate of the mean number of litters per day of gestation which the whole sample would have been expected to include for each day of gestation if 100% of the litters survived. It has been shown experimentally (Brambell *et al.* 1948) that when all the embryos in a litter are killed at $11\frac{1}{2}$–12 days post-coitum the age of embryos can be determined for a maximum of 3–$3\frac{1}{2}$ days after death. This is the maximum period under favourable experimental conditions when the autopsy is performed immediately after the death of the mother, before putrefaction is superimposed on the autolysis of the embryos that has been proceeding during her life. Since the autopsies on the wild material were performed on the average many hours after the death of the mother it is improbable that the age of embryos, which had been reabsorbing for so long a period before her death, would still have been determinable at the time of autopsy. It will be a reasonable, but conservative, estimate if the lower figure of 3 days, therefore, is taken as the maximum for which, on the average, the age of embryos dying between the 9th and 15th days post-coitum inclusive, would remain determinable in the wild material under the given conditions. The proportion of litters lost ($=L$) can be calculated from the formula

$$L=n/Km, \qquad \text{(xii)}$$

where $n=$the number of reabsorbing litters obtained, $m=$the mean number of litters obtained per day of gestation, and $K=$the period in days during which the age of the embryos in a dead litter can be determined. Since eighty-nine reabsorbing litters of these ages were obtained the estimated proportion of litters lost by the 15th day is 0·3180. The experiments referred to have shown that when all the

embryos are killed at 16½ days post-coitum their age remains recognizable for a day longer. Since twelve reabsorbing litters were obtained that died on the 16th day or subsequent days of gestation these represent a further loss of litters, estimated in the same way, of 0·0322. Thus the total loss after implantation of litters that are reabsorbed would be 35 %. It should be noted that this method would not include any loss that may occur by abortion.

The third and the most direct method is to determine the relative frequency of animals in early stages of pregnancy and of those in late stages, after the litters have been lost. This method is open to the objection that the methods used to obtain the material, trapping, shooting, etc. may have been selective in favouring the chances of obtaining animals at certain stages of pregnancy as compared with others, so biasing the results. For example, animals approaching full term may be more sedentary in habit and hence less liable to be trapped or shot, or, conversely, their greater weight may favour the chances of their springing a trap and being caught. Animals in which the uterus was damaged in cleaning and in which the full number of healthy embryos and implantation sites was not known had to be excluded from the mortality tables. Amongst these, animals approaching full term predominated so that it is necessary to include them all for the present purpose. A total of 764 animals was obtained 0–7 days pregnant inclusive, giving a mean number of 95·5 per day for the 8-day period. A total of 775 animals was obtained 21 days pregnant and over. This is an 11-day period since the young are born on the 31st or 32nd day and so gives a mean number of 70·45 per day. The difference represents a disappearance of 26·2 % of litters. Since it is the sample of animals approaching full term which is most liable to be biased by selective trapping, etc. it would appear to be preferable to confine the calculation to those 21–25 days pregnant, of which 308 were obtained, a mean number of 61·6 per day. The difference from the 0–7-day group on this basis represents a loss of 35·5 % of litters.

It would appear safe to conclude that not less than 35 % of litters are lost as such after implantation and that the proportion may be as high as 56 % or even slightly more.

Although the duration of the phase of reabsorption from the time of disappearance of the embryos to the time when the reabsorption sites cease to be recognizable as such has been determined experimentally (Brambell *et al.* 1948), late reabsorption stages have not been used for estimating the proportion of litters lost. This is because many animals appear to become pregnant again before reabsorption is complete, and then soon cease to be recognizable as late reabsorption stages. Several such pregnant animals with still recognizable reabsorption sites dating from a preceding pregnancy were observed and it is probable that many others were overlooked. Although the late phase of reabsorption lasts about twice as long as the phase during which the embryos are ageable in experimental animals, it can be seen from Table 2 that 101 animals in the earlier phase were obtained as compared to only sixty-three in the later phase, clearly indicating substantial wastage from the latter group.

The relation of the loss after implantation to the body weight

The body weight is related both to the age and condition of the animal and hence it is necessary to examine how the loss after implantation varies with the weight of the mother. The loss in surviving litters, examined at all stages from implantation to full term, for each 100 g. body-weight group is summarized in the second column

Table 7. *Loss after implantation according to body weight*

Body weight in g.	Proportion of embryos lost in 7–32-day age group of living litters	Proportion of embryos lost in 11–15-day age group of living litters	Proportion of all litters dead
1400 <	0·0805 ± 0·0067	0·2238 ± 0·0205	0·0251 ± 0·0093
1300–1399	0·0789 ± 0·0053	0·2398 ± 0·0176	0·0593 ± 0·0107
1200–1299	0·0737 ± 0·0050	0·2598 ± 0·0200	0·0793 ± 0·0114
1100–1199	0·0712 ± 0·0060	0·2244 ± 0·0220	0·1060 ± 0·0151
1000–1099	0·0676 ± 0·0095	0·2571 ± 0·0370	0·1381 ± 0·0257
< 999	0·0909 ± 0·0204	0·2632 ± 0·0714	0·2090 ± 0·0497

of Table 7. The proportion of dead embryos provides the best measure of such loss. The standard deviation has been calculated from the formula

$$\sigma = \sqrt{(pq/n)}, \qquad \text{(xiii)}$$

where p = the proportion of dead embryos, q = the proportion of surviving embryos, and n = the number of implantation sites. This method is open to the objection that only part of the mortality is known to approximate to a binomial distribution. It will be seen that the proportion of embryos lost rises steadily with increasing body weight from 1000 g., but that even the extreme values do not differ significantly from each other.

Since the majority of litters in process of being lost are concentrated in the 11–15-day age group the data for this age group have been examined separately and are summarized in the third column of Table 7. They reveal no significant relation to body weight.

The body-weight distribution of the litters in which all the embryos are dead and reabsorbing has been examined also, and the data are summarized in the right-hand column of Table 7. The proportions of dead litters, calculated as proportions of all litters surviving and dead, with their standard deviations, are given and are represented graphically in Fig. 5. It is apparent that the proportion of dead litters falls steeply and consistently with increasing body weight and that the relation is clearly significant. It must be remembered that the proportions of dead litters expressed in this way does not provide reliable information as to the actual proportion of litters lost, because the time during which reabsorption sites remain recognizable after the deaths of the embryos is not known accurately, yet this does not affect their relative significance. It must be concluded, therefore, that the proportion of

litters lost *in toto* after implantation declines steeply with increasing body weight of the mothers.

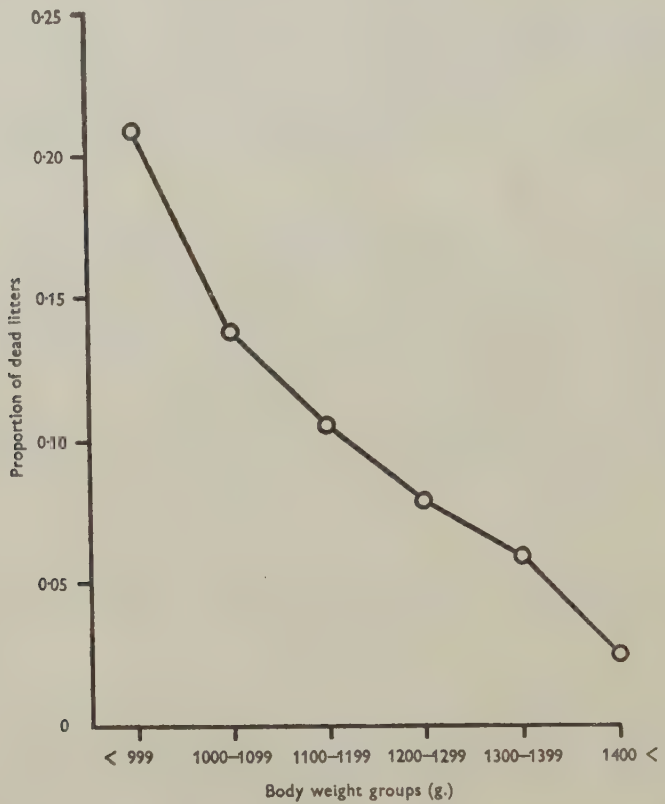


Fig. 5. The proportion of dead litters represented as a proportion of all living and dead litters according to the cleaned body weight of the mother.

The relation of the loss after implantation to whether milk is present or not in the mammary glands

It has been stated already (Allen *et al.* 1947) that only the presence or absence of milk in the mammary glands was recorded up till and including February 1942 (Series 01, 0 and part of 1). Thereafter, those with milk in the mammary glands were further subdivided into those with milk but not certainly suckling and those certainly suckling, as indicated by copious milk-secretion, large size of the mammary glands and depleted fur around the nipples. The three categories were recorded under the headings of no-milk, milk and lactating respectively. All animals 28 days pregnant and over were excluded since milk appears in the mammary glands at that stage of gestation irrespective of the previous history of the mother. The proportions of embryos lost after implantation, according to whether or not milk is present in the mammary glands for the two periods before and after the end of February 1942, are compared in Table 8. It is clear that there is no significant difference between the

proportions of embryos lost in the two periods, nor between the groups with no-milk and those with milk or lactating combined.

Table 8. *Proportion of embryos lost after implantation according to whether milk is present or not in the mammary glands*

Series	No-milk		Milk or lactating		All combined	
	Total no.	Proportion of embryos lost	Total no.	Proportion of embryos lost	Total no.	Proportion of embryos lost
01, 0 and 1 (7. ii. 41 to 28. ii. 42)	289	0·1038 ± 0·0179	550	0·0945 ± 0·0125	839	0·0977 ± 0·0102
1–8 (1. iii. 42 to 31. xii. 44)	959	0·0855 ± 0·0090	6494	0·0855 ± 0·0035	7453	0·0855 ± 0·0032
All combined	1248	0·0897 ± 0·0081	7044	0·0862 ± 0·0033	8292	

Table 9. *Proportion of embryos lost after implantation according to whether milk is present or not in the mammary glands in each age group*

Age-group (days)	No milk		Milk		Lactating	
	Total no.	Proportion of embryos lost	Total no.	Proportion of embryos lost	Total no.	Proportion of embryos lost
7–10	169	0·0118 ± 0·0083	709	0·0536 ± 0·0084	682	0·0279 ± 0·0063
11–15	252	0·1905 ± 0·0248	754	0·1950 ± 0·0144	766	0·2950 ± 0·0165
16–20	227	0·0617 ± 0·0160	798	0·0401 ± 0·0070	878	0·0740 ± 0·0088
21–27	311	0·0579 ± 0·0132	1434	0·0153 ± 0·0032	473	0·0127 ± 0·0051
Total	959	0·0855 ± 0·0090	3695	0·0647 ± 0·0040	2799	0·1129 ± 0·0060

The results are different when for the second period the groups with milk and lactating are separated and are compared with each other and with the no-milk group, as is done in Table 9. The difference between the proportions of embryos lost in the sample with no-milk and in each of the samples with milk and lactating respectively is just over twice its standard error and so is significant, though barely so. The difference between the proportions of embryos lost in samples with milk and lactating respectively is nearly seven times its standard error and is very significant. The loss is greatest in the lactating group and least in the milk group. Comparison of the proportions of embryos lost in each age group of each category reveals further differences. The proportion of embryos lost in the 11–15-day age group, in which the litters in process of being lost entirely tend to be concentrated, is obviously much greater in lactating animals, than in either of the other two categories, and this accounts for the heavier mortality in the lactating animals treated as a single sample. There is clearly no significant difference between the proportions of embryos lost in the no-milk and milk categories respectively for this age group. The difference between the proportions of embryos lost in the no-milk and lactating categories in the 7–10-day age group is not significant, but both are significantly less than in the milk category. The proportion of embryos lost in the no-milk category does not differ significantly from that in either of the other categories for the 16–20-day age group, but the proportion of embryos lost in the lactating animals is

significantly higher than that in the animals with milk. The proportions of embryos lost in the milk and lactating categories for the 21–27-day age group do not differ significantly, but both are significantly less than in the no-milk category.

Geographical and annual variation in the incidence of loss after implantation

Comparison of the incidence of the mortality after implantation in the different series should show (*a*) whether it varies geographically from one locality to another in Great Britain, and (*b*) whether it varies, from one year to another in the same locality. The data for all pregnancies in the 7–32-day age group for each series are summarized in the second column of Table 10. The proportion of embryos lost in Series 4 is

Table 10. *Loss after implantation in the different series*

Series	Proportion of embryos lost in 7–32-day age group of living litters	Proportion of embryos lost in 11–15-day age group of living litters	Proportion of all litters dead
0+01	0·0800±0·0095	0·2360±0·0335	0·1182±0·0227
1	0·0795±0·0072	0·2882±0·0267	0·1235±0·0183
2	0·1069±0·0132	0·2458±0·0322	0·0650±0·0222
3	0·0554±0·0058	0·1551±0·0143	0·0417±0·0113
4	0·1413±0·0097	0·4558±0·0296	0·1377±0·0197
5+6	0·0534±0·0054	0·1549±0·0192	0·0407±0·0107
7+8	0·0583±0·0048	0·1951±0·0197	0·0594±0·0120

greater than in any other, and differs significantly, though barely so, from the next greatest in Series 2. The best comparison geographically is between Series 3 and 4, from Anglesey and Caernarvonshire respectively, because these two series were taken simultaneously in 1944, the weekly samples of each being dealt with alternately, and hence the personal factor in arbitrary determinations and in technique was at a minimum. Yet the difference between the proportions of embryos lost in these two series is over seven times its standard error and is clearly significant. Comparison of Series 1 and 4, both from South Caernarvonshire, collected in 1942 and 1944 respectively, is the best for analysis of annual variation, the difference between the proportions of embryos lost being five times its standard error. The difference between the proportions of embryos lost in Series 2 and 3, both from Anglesey in 1943 and 1944 respectively, is also significant, being over three times its standard error, but is less satisfactory as an example of annual variation since the two series were obtained from different parts of the country and the variation might be geographical.

Since litters in process of being lost entirely tend to be concentrated in the 11–15-day age group, comparison of the incidence of the loss after implantation in the different series in this age group should throw light on which fraction of the total loss of embryos varies geographically and annually. The data are summarized in the third column of Table 10. Analysis reveals corresponding differences of similar significance, justifying the conclusion that the incidence of loss of whole litters after implantation varies both geographically and annually.

Information on these points can be obtained also from the numbers of reabsorbing litters in which all the embryos were dead, and which, therefore, are not included in the above data. The data are summarized in the right-hand column of Table 10, and are treated in a similar manner to that in the section on the relation of the loss to body weight. Analysis reveals significant geographical variations in the proportion of reabsorbing litters obtained, but the annual variations are not significant. Nevertheless, the results correspond remarkably well as regards the relative incidence of the loss of whole litters in the different series to those derived from the surviving litters. The results of all three analyses are compared graphically in Fig. 6, and it can be seen that in all the incidence of mortality is highest in Series 4 and lowest in Series 7+8, 3, and 5+6 in descending order.

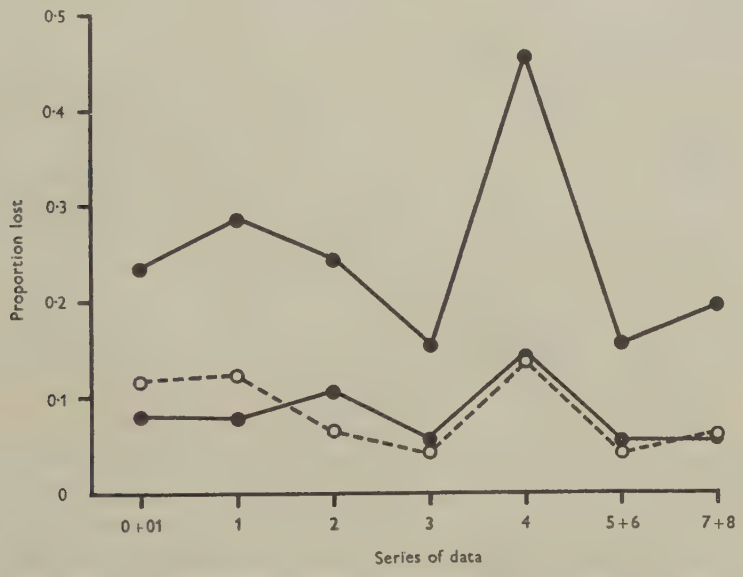


Fig. 6. The mortality in each series of data is represented. The solid circles represent the proportions of embryos lost, the upper ones in the 11–15-day age group only and the lower ones in the whole sample of pregnancies (7–32 days). The hollow circles represent the proportions of dead litters expressed as proportions of all living and dead litters.

Seasonal variations in the loss after implantation

The data were grouped into 4-week periods and are summarized in Table 11. The results, which are represented graphically in Fig. 7, are not very satisfactory. It appears that the incidence of mortality attains maximal values in the 2nd and 6th 4-week periods, that is, at the beginning and end of the intensive breeding season. Both these peaks are significant for the 11–15-day group, though barely so, the former is significant in the data for the 7–32-day group but not in those for dead litters, and the latter in the data for dead litters but not in those for the 7–32-day group. Probably both maxima are due mainly, if not entirely, to a greater incidence of loss of whole litters at these periods.

Table 11. *Loss after implantation for successive 4-week periods*

Weeks	Proportion of embryos lost in 7–32-day age group of living litters	Proportion of embryos lost in 11–15-day age group of living litters	Proportion of litters dead
1–4	0·0444 ± 0·0116	0·1091 ± 0·0297	0·1047 ± 0·0330
5–8	0·0954 ± 0·0086	0·2587 ± 0·0259	0·0799 ± 0·0160
9–12	0·0713 ± 0·0056	0·2111 ± 0·0189	0·0668 ± 0·0118
13–16	0·0676 ± 0·0047	0·2090 ± 0·0177	0·0818 ± 0·0117
17–20	0·0775 ± 0·0052	0·2936 ± 0·0210	0·0742 ± 0·0121
21–24	0·0972 ± 0·0132	0·4384 ± 0·0581	0·1579 ± 0·0342
25–52	0·1000 ± 0·0254	0·2571 ± 0·0739	0·1026 ± 0·0486

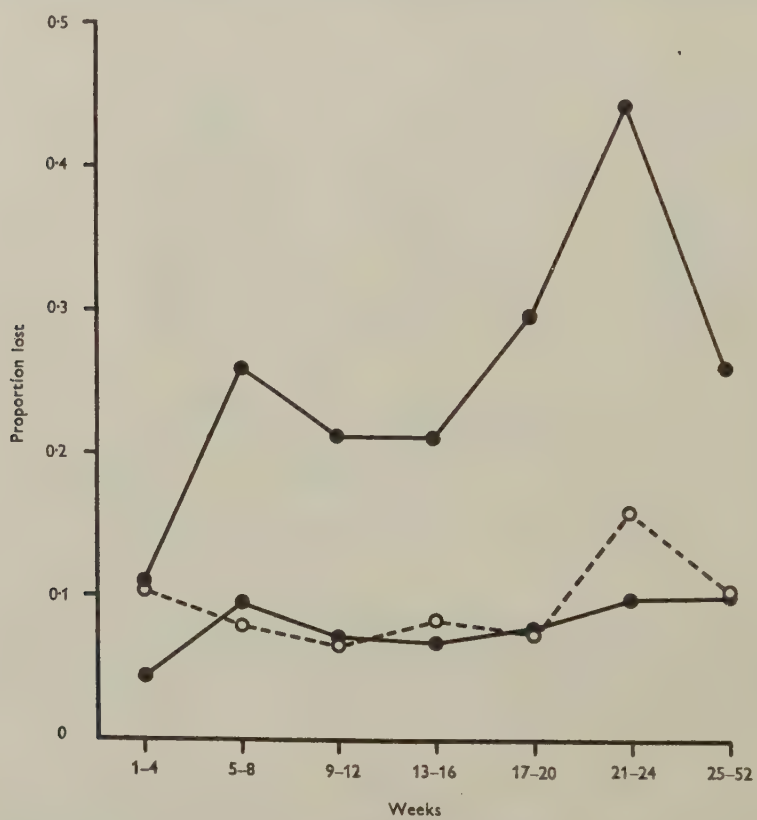


Fig. 7. The mortality for successive 4-week periods throughout the breeding season is represented. The solid circles represent the proportions of embryos lost, the upper ones in the 11–15-day age group only and the lower ones in the whole sample of pregnancies (7–32 days). The hollow circles represent the proportions of dead litters expressed as proportions of all living and dead litters.

Loss before and after implantation in litters surviving to near full term

It is not possible to determine the total prenatal mortality suffered by surviving litters since no data are available of the number of newborn young. The mortality at parturition therefore is unknown. An estimate of the total mortality prior to

parturition in surviving litters can be obtained from the 26-day-and-over age group and the data for the combined loss before and after implantation in this age group are contained in Table 12. It is apparent that 41 % of the litters have suffered some loss and that 11 % of the ova ovulated have been lost, but it is probable, for the reasons given previously (Allen *et al.* 1947), that this is an underestimate owing to an error arising through the omission of corpora lutea and of very old reabsorption sites from the counts.

Table 12. *Loss before and after implantation in litters surviving to near full term (26–32 days)*

No. of ova ovulated	No. of ova lost						Litters		Ova	
	0	1	2	3	4	5	Total no.	No. showing loss	Total no.	No. lost
11	—	1	—	—	—	—	1	1	11	1
10	1	1	—	2	—	—	4	3	40	7
9	3	2	1	1	—	1	8	5	72	12
8	9	11	7	2	—	—	29	20	232	31
7	29	20	8	2	1	—	60	31	420	46
6	66	37	12	2	3	—	120	54	720	79
5	68	28	3	2	4	—	105	37	525	56
4	70	18	8	3	—	—	99	29	396	43
3	15	4	1	—	—	—	20	5	60	6
	261	122	40	14	8	1	446	185	2476	281

Proportion of litters showing loss $0{\cdot}4148 \pm 0{\cdot}0234$. Proportion of ova lost $0{\cdot}1135 \pm 0{\cdot}0064$.

DISCUSSION

The significance of the separation of the data of loss before implantation from those of loss after implantation is worth stressing again. The former can be derived from comparison of the number of corpora lutea in the ovaries with the number of implantation sites in the uterus, so far as litters that have survived implantation are concerned, and the latter from comparison of the number of implantation sites with the number of surviving embryos. This is so obvious, when attention is directed to it, that its importance may easily be overlooked, yet it is a most valuable procedure in the analysis of prenatal mortality. Not only has the prenatal mortality in each of these two fractions been shown to be mutually independent (Allen *et al.* 1947), but the two fractions are not comparable and are subject to different limitations. The data for loss before implantation are derived entirely from litters which have survived implantation and, therefore, they do not include litters which have been lost *in toto* before implantation but do include all the loss in litters that survived implantation, because the loss is then complete. The data for loss after implantation, being derived from pregnant uteri at all stages between implantation and full term, concern loss in progress which, therefore, is not complete but which does include litters in process of being lost *in toto* at the time of autopsy. Loss at parturition is necessarily excluded. Further, the data for loss before implantation, being derived from animals in which this loss has been completed, provide no information regarding the precise

stage between ovulation and implantation at which the loss occurred, whereas the data for loss after implantation, since they are derived from animals at known stages of pregnancy in which the loss is in progress, do provide information regarding the incidence of the loss at successive stages after implantation. These are fundamental differences of great significance.

It has been shown that the distribution of the loss will vary according to whether the mortality is falling on the embryos as units or on the litters as units. The relation of the proportion of litters showing loss to the proportion of embryos lost provides a means of distinguishing between these two kinds of mortality. Assuming that the proportion of embryos lost is constant, irrespective of litter size, then the proportion of litters showing loss will increase geometrically with increasing litter size if the loss is falling at random on the embryos as units, whereas it will remain constant if the loss is falling at random on the litters as units. The loss before implantation, which necessarily excludes loss of whole litters, approximates closely to the expectation for a mortality falling at random on the embryos as units (Brambell & Mills, 1947*b*, fig. 1). A theoretical distribution that provides both for a loss falling at random on litters as units and for a loss falling at random on the embryos as units in the surviving litters has been worked out and provides a good fit for the data of loss after implantation. Comparison of the figure referred to with Fig. 1 illustrates this point clearly. The whole of the increase in the proportion of litters showing loss with increasing litter size observed can be accounted for by that fraction of the mortality which falls on the embryos as units. Therefore there is no evidence that the proportion of litters lost *in toto* after implantation increases with increasing litter size, and indeed the data of reabsorbing litters in which all the embryos are dead indicate that it declines. One of us in an earlier paper (Brambell, 1944), before the necessity of distinguishing between the loss before and after implantation was appreciated, concluded erroneously that the proportion of litters lost *in toto* did increase with litter size and deduced therefrom that, in consequence, litters of 5 and 6 were more productive than larger or smaller litters (Brambell, 1944, text-fig. 14). This we have now shown to be incorrect and to have been due to the methods of analysis then employed not enabling the mortality falling on the embryos as units to be distinguished from that falling on the litters as units.

The problem of estimating the proportion of litters lost after implantation presents great difficulty, chiefly because of uncertainty in the time factors involved. No precise information is available as to the time elapsing between the deaths of the first and last embryos in a litter, and as to whether this varies according to the number of embryos. Differences in the stages of development attained by the embryos seldom exceed the equivalent of 1 day, but this may be an unreliable guide since it is quite possible, indeed probable, that development may be retarded when the litter is dying. These factors affect estimates based on the proportion of litters in process of dying and which are therefore liable to error. Similarly, the time elapsing between the death of the last embryo in a litter and the point at which autolysis has proceeded so far that the stage of development it had attained at death can no longer be determined is not known precisely. Since no information could be obtained from

the literature as to the duration of the reabsorptive processes in the rabbit, a separate experimental investigation of this problem was undertaken by killing all the embryos in tame rabbits at various stages of development, either by direct surgical interference or by injection of massive doses of stilboestrol (Brambell *et al.* 1948). Since, however, the time was found to vary both with the experimental method employed of killing the embryos and with the stage of development which they had attained, the results are not entirely adequate, since the cause of death of the embryos in the wild rabbits is unknown and may affect the duration of the reabsorptive processes. The estimates, based on the proportion of dead litters obtained, depend on this time factor, and it has been necessary to employ that derived from the experiments. The third method of estimating the proportion of litters lost, based on the relative numbers of animals in early and late stages of pregnancy obtained, is liable to error arising from selective trapping, as has been indicated. Thus all three methods are liable to errors, which may be large. The first method gives an estimated loss of 56 % of the litters, the other two methods agree in giving an estimated loss of 35 % of the litters. Probably the actual loss lies between these values.

Another difficulty in estimating the total loss of litters arises from the fact that 5 % more embryos are reabsorbing in the 16–20-day age group than in the 26–32-day age group. This can be accounted for only either by the total loss of some of the litters in the intervening period, which therefore are not represented in the 26–32-day age group, or by the disappearance of some of the old reabsorption sites in surviving litters before the 26th day, thus reducing the apparent mortality in the 26–32-day age group. However, the proportion of dead litters in which all the embryos are reabsorbing obtained after the 16th day is too small to account for the necessary loss of whole litters, hence it must be concluded that if whole litters are lost at this stage they must be aborted, not reabsorbed. This conclusion is supported by the experimental evidence (Brambell *et al.* 1948) that abortion is a more frequent means of removal of a dead litter after the 19th day in rabbits than reabsorption. On the other hand, it has been shown (Allen *et al.* 1947) that the mean number of implantation sites in the uteri declines from $5{\cdot}42 \pm 0{\cdot}07$ at 16–20 days to $4{\cdot}92 \pm 0{\cdot}07$ at 26–32 days, an observation which is difficult to account for otherwise than by assuming that 9 % of the old reabsorption sites escape recognition, and are omitted from the counts, in late stages of pregnancy. If so, this error would be more than sufficient to account for the apparent excess of the mortality in the 16–20-day age group as compared to the 26–32-day age group. Of these alternative explanations, the former would have the effect of increasing the estimated loss of whole litters between the 11th and 15th days by the proportion aborted subsequently, whereas the latter would increase the mortality suffered by surviving litters above that apparent in the 26–32-day age group, as shown in both Tables 4 and 12.

Although, for the reasons set out above, estimates of the absolute proportions of litters lost and of embryos lost in surviving litters after implantation must be treated with caution and are likely to be subject to a wide margin of error, the same considerations do not apply to comparisons of the observed proportions either of embryos lost or of dead litters in the various sub-samples. The relative significance

of these values for the various sub-samples remains unaffected, assuming that the times taken for a litter to die and to be reabsorbed tend to be constant. The remarkable consistency of the data justify this assumption. Analysis of the relation of the loss after implantation to body weight of the mother, to the functional condition of her mammary glands, to the locality, to the year and to the season therefore have been confined to such direct comparisons. Thus it has been possible to determine that the proportion of litters lost is inversely related to the body weight of the mother; that is, the loss is heavier in the younger and less well-conditioned animals as compared to the older and better conditioned animals. This is shown very clearly by the proportion of dead litters. The mortality varies also with the functional activity of the mammary glands, being heaviest in animals that were certainly suckling and least in those that had some milk in the glands. There is some indication that the mortality tends to occur rather earlier in gestation in the animals with some milk and later in those with no milk than in the undoubtedly suckling animals. The proportion of litters lost also varies significantly both in different localities in the same year and in different years in the same locality. Finally, the mortality appears to be heavier at the beginning and end than during the height of the breeding season.

SUMMARY

1. Data of prenatal mortality occurring after implantation are derived either from (*a*) comparison of the number of implantation sites with the number of surviving embryos in pregnant uteri, or (*b*) the proportion of animals with nothing but dead and reabsorbing embryos in the uteri. The theoretical limitations to which such data are subject are examined and are found to be very different from those applicable to data of mortality occurring before implantation.

2. The proportion of embryos lost in the whole sample of 1834 litters ranging in age from implantation to full term (7–32 days) varies according to the size of litter at implantation as the linear regression

$$Y = 0{\cdot}00084x + 0{\cdot}0709,$$

where x = the number of implantation sites. The proportion of litters showing loss varies similarly as

$$Y = 0{\cdot}02093x + 0{\cdot}0866.$$

It is shown that the relation of the proportion of embryos lost to the proportion of litters showing loss does not accord with the assumption that the whole loss is falling at random upon the embryos as units, but that part of the loss must be falling on the litters as units. Such a mortality theoretically would be distributed as

$$E = \tfrac{1}{2}l + p(1 - l),$$

and

$$L = lq^x - q^x + 1,$$

where E = the proportion of dead embryos, L = the proportion of litters showing loss, l = the proportion of litters in process of being lost as units, p = the proportion of embryos lost as units, $q = 1 - p$, and x = the number of implantation sites. This distribution provides a good fit for the data.

3. Altogether 164 animals were obtained in which all the embryos had died and were reabsorbing. The age at which the last embryo died could be determined in 101 of these. These data show that the greater the number of embryos which become implanted the less is the probability of the litter being lost, and that the majority of these litters were lost between the 11th and 15th days of gestation inclusive.

4. There is a corresponding maximum in the proportion of living litters containing some reabsorbing embryos between the 10th and 15th days of gestation inclusive. Not all the mortality in the 16–20-day age group of living litters in excess of that in later age groups can be accounted for by reabsorption of whole litters. Therefore, either some litters must be aborted at this stage or some old reabsorption sites must be overlooked in the counts at subsequent stages or both must occur

5. The proportions of litters lost as units and of embryos lost as units in surviving litters have been estimated in successive age groups of living litters. The estimated maximum proportion of litters in process of being lost *in toto* was 0·56 on the 12th day of gestation. The estimated proportion of litters lost, based on the proportion of living to dead litters obtained and allowing for the rate of reabsorption, was 0·35, but this estimate excludes any loss by abortion. The estimated proportion of litters lost, based on the relative frequency of animals obtained in early and late stages of pregnancy, was 0·355.

6. The proportion of litters lost *in toto* declines steeply with increasing body weight of the mothers.

7. The mortality varies with the mammary activity of the mothers, being greatest in those animals that were certainly lactating.

8. The incidence of the loss of whole litters varies both (*a*) from one locality to another in Great Britain, and (*b*) from one year to another in the same locality.

9. It is probable that the incidence of mortality is greater at the beginning and end of the breeding season than during its height.

10. An attempt is made to estimate the total mortality suffered both before and after implantation in those litters that survive to near full term. Of such litters not less than 41 % have suffered some loss and not less than 11 % of the ova ovulated have been lost.

REFERENCES

Allen, P., Brambell, F. W. Rogers & Mills, I. H. (1947). *J. Exp. Biol.* **23**, 312.
Brambell, F. W. Rogers (1942). *Proc. Roy. Soc.* B, **130**, 462.
Brambell, F. W. Rogers (1944). *Proc. Zool. Soc. Lond.* **114**, 1.
Brambell, F. W. Rogers, Henderson, M. & Mills, I. H. (1948). *J. Exp. Biol.* **25**, 209.
Brambell, F. W. Rogers & Mills, I. H. (1947*a*). *J. Exp. Biol.* **23**, 332.
Brambell, F. W. Rogers & Mills, I. H. (1947*b*). *J. Exp. Biol.* **24**, 192.
Minot, C. S. & Taylor, E. (1905). *Normentaf. Wirbelt.* **5**.
Reynolds, S. R. M. (1946). *Anat. Rec.* **95**, 283.

THE TOXIC ACTION OF COPPER AND MERCURY SALTS BOTH SEPARATELY AND WHEN MIXED ON THE HARPACTICID COPEPOD, *NITOCRA SPINIPES* (BOECK)

By H. BARNES and F. A. STANBURY
The Marine Station, Millport and The Technical College, Plymouth

(*Received* 31 *October* 1947)

INTRODUCTION

In connexion with the anti-fouling investigations by the Marine Corrosion Sub-Committee of the Iron and Steel Institute (now a Committee of the British Iron and Steel Research Association) the toxicities of a large variety of substances have been determined (reported by Harris, 1946). The primary object was a comparison of the behaviour of these substances in laboratory toxicity tests using a marine animal, with their anti-fouling performance when used in paint media. Copper and mercury salts were included in these tests, since compounds of these metals are commonly used in anti-fouling compositions. In the course of this work, tests were carried out on mixtures of copper and mercury salts as well as on the salts used alone. The results with these two heavy metal salts seem worth recording since, although no experiments were made to elucidate more fully the factors involved, no reference to such striking synergic phenomena in oligodynamic effects of heavy metals could be traced in the literature.

EXPERIMENTAL

A rapid and simple technique was required to deal with the large number of substances. The animal used was the Harpacticid Copepod *Nitocra spinipes* (Boeck). Animals were collected on the day of use and all the experiments quoted were done at the same time of the year. No attempt was made to select individuals of a particular size group; good agreement between the numerous replicates indicates that no considerable error had arisen from using mixed populations.

Ten animals were transferred to 5 ml. of the poison solution contained in small flat-bottomed dishes which were then covered with a glass plate. Inspection was made after 6 and 24 hr., when the animals were classified as dead, moribund or living. There was little difficulty in deciding when the animals were dead since they then showed a characteristic dorsal flexure of the urosome. Replicates (using ten animals for each test) were carried out so that for any one concentration or combination of concentrations the figures given in Table 1 represent the mean of observations on approximately 100 animals. Controls were invariably living at the end of the test period.

Sea water was collected from outside the Plymouth Breakwater and stored in carboys. The mercuric chloride (A.R.) solutions were made up in sea water and diluted as required: the copper sulphate (A.R.) solutions were made up initially in distilled water to a concentration of 10 g./l., with subsequent dilutions in sea water.

RESULTS

Table 1 shows the percentage of animals killed by the two poisons alone and admixed. Only the results for the 24 hr. inspection are quoted, since those for the 6 hr. period showed the same trend.

Table 1

Concentration (mg. Cu/l.) \ Concentration (mg. Hg/l.)	0	0·07	0·15	0·31	0·40	0·60	0·70	1·5	3·0	4·4
0	0	0	1·4	10·0	16·7	50	72	78	84	100
0·026	1·3	9·1	14·5	12·7	50·0	61·8	76·4	87·3	100	100
0·26	11·3	11·9	20·0	45·6	93·7	100	100	100	100	100
2·6	21·2	—	78	82	98	100	100	100	100	100
26	42·5	—	—	—	—	—	—	—	—	—

DISCUSSION

Solubility considerations of copper and mercury

In any consideration of these results the state of the metals in solution in the presence of dissolved salts will be of importance. The solubility of mercuric chloride in distilled water is 72·2 g./l. at 25° C. The dissociation is said to proceed in two stages, Morse (1902):

$$HgCl_2 \rightleftharpoons HgCl^+ + Cl^-,$$

$$HgCl^+ \rightleftharpoons Hg^{++} + Cl^-.$$

Sherrill (1903 & 1904) considers that the complex ion $HgCl_4^=$ is also present, and in addition there are indications that polymerization takes place.

$$xHgCl_2 \rightleftharpoons (HgCl_2)x.$$

The solubility in chloride solution increases with increasing chloride content (Thomas, 1939) and in sea water is of the order of 270 g./l. at 25° C. In these chloride solutions a number of complex ions have been postulated and some evidence obtained for their existence. In saturated solutions of the salt in chloride solutions Garrett (1939) has indicated that the dominant ion is that of $HgCl_3^-$, accompanied by very small amounts of $HgCl_4^=$ and complex ions, such as $(HgCl_2)Cl^-$, formed from the polymers. In view of the lack of agreement between various workers it is not possible to calculate the quantities of the various entities present, but in very dilute solutions polymerization might be expected to be small, and calculations based on any of the data suggest that there are only traces of the mercuric ion present and that the major entities are undissociated mercuric chloride

and complex ion. For example, if Sherrill's equilibrium constants are used, the solution for median lethal dose (0·6 mg. Hg/l.) contains

$$Hg^{++} = 4 \times 10^{-5} \text{ mg. Hg/l.},$$

$$HgCl_4^{=} = 0{\cdot}46 \text{ mg. Hg/l.},$$

$$HgCl_2 = 0{\cdot}14 \text{ mg. Hg/l.}$$

It is also of interest to note that the work of Le Blanc and Noyes and Richards and Archibald (see Garrett, 1939), indicates that the complex is a highly stable strong electrolyte.

The solubility relations of the cupric ion in sea water are no less complex. When metallic copper is immersed in sea water in the presence of an adequate supply of oxygen a blue-green precipitate is formed. Bengough & May (1924) considered the product to be a mixture of basic cupric carbonate and chloride, predominantly the former. The concentration of copper in equilibrium with such a freshly precipitated 'basic copper carbonate' is of the order of 0·5 mg./l., but if the precipitate is allowed to age in contact with the solution, its solubility is reduced to the order of 0·1 mg. Cu/l., suggesting that there may be a slow conversion to a true atacamite (basic cupric chloride) (Ferry & Riley, 1946). Free (1908) has shown that the solubility of 'basic copper carbonate' decreases on contact with water containing carbon dioxide, and is increased in the presence of chlorides. The solubility product $[Cu^{++}][OH^-]^2$ in equilibrium with solid cupric hydroxide has been given as $1{\cdot}6 \times 10^{-19}$ (Feitknecht, 1944). At pH 8·0 this would correspond to a copper concentration of approximately $0{\cdot}6 \times 10^{-4}$ mg. Cu/l. (Taking the ionic strength of sea water as 0·63 and the activity of divalent cupric ion in such a solution as 0·17.) This is much less than the experimental values already given for the solubility of the 'basic copper carbonate'.* Further, when solutions of copper sulphate are added to sea water, no immediate evident precipitation takes place at concentrations far in excess of either the solubility of the hydroxide or even the experimentally determined values for the 'basic carbonate'. Even with high copper concentrations it has been found that precipitation may be delayed for many hours. This appears to have been the experience of other workers. The above figures would suggest that the copper is not present as cupric ion and other possibilities have to be considered.

The formation of complex ions is indicated but their character is not clear. Ammonia readily forms complexes with the cupric ion but, since the ammonia concentration is only of the order of $0{\cdot}02 \times 10^{-5}$ mg./l., it can play only a small part when this value is considered in relation to the data of Stackelberg & Freyhold (1940) for the association constant of the first two complexes of the cupric ion and ammonia. Basic compounds other than atacamite have been reported, whose solubility product may be higher, and complex double salts with alkali chlorides and sulphates are known.

It would appear that on the rapid addition of a copper sulphate solution of moderately high concentration to sea water complex ions are formed, including

* The possibility of the precipitation of copper phosphate has been neglected since it would not affect the present argument.

small concentrations of cations such as $[Cu^{++}][NH_3]$ and $[Cu^{++}][NH_3]_2$ with ammonia, and anions with the chlorides or even sulphates present; these slowly react with the carbonate and hydroxyl ions to produce extremely insoluble basic salts. The latter may exist in a colloidal form before aggregating to give a precipitate.

A further contributory factor may be the lowering in pH which takes place moderately rapidly on storing sea water and which would allow a higher concentration of cupric ions to be present.

It remains to consider the toxic action of the two metals in solution.

The toxic action of the copper and mercury solutions

It is evident that mercuric chloride is a very effective poison, the median lethal dose being of the order of 0·6 mg. Hg/l. In contrast, copper sulphate does not kill 50% of the animals at a 'concentration' of 26 mg. Cu/l., and a tenfold increase in 'concentration' from 0·26 to 2·6 mg./l. only results in an increase of from 11 to 21% in the kill. These results suggest that the mode of action of the two salts may be different. It is possible, however, that the slow increase in effective action with increasing copper concentration may be due to the formation of colloidal basic compounds, whose poisonous action may be entirely different from the cupric or cupri-complex ion.

In investigations of fresh-water animals Jones (1937), extending the work of Carpenter (1927), has shown that heavy metal poisoning (including that by copper and mercury) of the minnow, *Phoxinus phoxinus*, is due to asphyxiation following coagulation of the gill mucus. In addition, respiration studies by the same worker (1941–2) on *Gammarus pulex* and *Polycelis nigra* gave results which indicate that both metals acted on these two animals in a similar manner. Jones (1941) has also shown that $HgCl_2$ alone, and in the presence of NaCl, does not differ in toxicity to the minnow. Comparative work, using a brackish form and investigating the effect of these metals at variable salt content, would be valuable in this respect: they might be expected to reveal that mercuric salts, due to a considerable concentration of undissociated $HgCl_2$ in either fresh or saline solutions, would show a similar behaviour, whilst that of copper solutions would vary with salinity due to the complex solubility relations in sea water. Mr K. A. Pyefinch and Mr W. Russell Hunter (personal communication), working with *Marinogammarus marinus*, have recently shown that changes in the nature of copper poisoning with variations in salinity take place which are in accordance with the above suggestion. Further, it is of interest to note that Clarke (1947) found that 'basic copper carbonate' was more toxic to barnacles than the complex citrate or tartrate, although the difference was not striking, and that Miller (1946) states that Carritt and Riley were unable to find any difference between cupric and cuprous copper poisoning. It has been established that cuprous salts exist in sea water largely as complex cupro-chloride ions.

The combined effect of copper and mercury solutions

It is clear from Table 1 that mixtures of these poisons are often far more effective than either poison alone at the same concentrations and, moreover, that toxicity is frequently far greater than would be expected on an additive basis.

These results strengthen the hypothesis that the two metals behave differently towards the animal, and that different systems or at least different parts of the same system are attacked. The simplest explanation of the synergy would appear to be that lowered vitality, due to one type of poisoning, does not allow the animal to deal as effectively with the second poison as when the latter is used alone. Thus impairment of the excretory system by mercury poisoning may allow sufficient accumulation of copper to interfere with the respiratory system which is thought by many workers to be involved in copper poisoning. Clarke (1947) has recently shown that barnacles and mussels can accumulate and excrete quantities of copper, and that even after considerable exposure to copper the animals can recover on return to normal conditions. He suggests that this metal acts slowly and not so much by destruction of some particular tissue or vital material as by a general retardation of vital processes. Thus the poisoning action of mercury may impair the excretory function and permit larger concentrations of copper to be built up.

Alternatively, the effect of one poison could change the permeability characters of the animal or cell membranes and so facilitate the entry of the second poison. In view of its solubility in organic solvents undissociated $HgCl_2$ might be expected to enter the cell rapidly, but its effect in coagulating any protein constituent might considerably change the permeability to ionic copper.

There is also the possibility that the effect is one external to the animal and not concerned with poisoning processes. Heavy metals, and particularly copper and mercury, are readily adsorbed on colloidal material; during the course of toxicity experiments a proportion of the poison in the external solution will be adsorbed on such material already present, and also on material secreted into solution during the course of the experiment (perhaps in increased amount in response to the poison). The amount available for poisoning might, therefore, be only a fraction of that added or estimated by analysis. In mixed poison solution preferential adsorption of one entity could allow a greater concentration of a second to be available for poisoning action.

It is clear that the problem of the toxic action of copper and mercury salts is one of great complexity. The above discussion is intended to draw attention to some of the physico-chemical equilibria involved in such toxic solutions in sea water, and also to possible variations in the mode of action upon the animal of the toxic compounds used. It is clearly very desirable to have more experimental work.

SUMMARY

1. The results are given of the poisoning of *Nitocra spinipes* (Boeck) by copper and mercury salts used together and separately.

2. The state of copper and mercuric salts in sea water is examined using the available physico-chemical data.

3. The results suggest that the two poisons act in a different manner and possible reasons for this are considered.

4. The striking synergic effects obtained when the two metals are used together are considered to support the suggestion in 3 above and various possible explanations, both biological and chemical for this synergism, are examined.

We wish to thank Prof. J. E. Harris for both his valued criticisms as well as his interest in the work.

REFERENCES

Bengough, G. D. & May, R. (1924). *J. Inst. Met.* **32**, 81.
Carpenter, K. (1927). *Brit. J. Exp. Biol.* **4**, 378.
Clarke, G. L. (1947). *Biol. Bull. Woods Hole*, **92**, 73.
Feitknecht, W. (1944). *Helv. chim. Acta*, **27**, 771.
Ferry, J. D. & Riley, G. A. (1946). *Industr. Engng Chem.* **38**, 699.
Free, E. E. (1908). *J. Soc. Chem. Ind., Lond.*, **30**, 1366.
Garrett, A. B. (1939). *J. Amer. Chem. Soc.* **61**, 2744.
Harris, J. E. (1947). *J. Iron Steel Inst.* 1946, 297.
Jones, J. R. E. (1937). *J. Exp. Biol.* **14**, 351.
Jones, J. R. E. (1940). *J. Exp. Biol.* **17**, 325.
Jones, J. R. E. (1941–2). *J. Exp. Biol.* **18**, 153.
Miller, M. A. (1946). *Biol. Bull. Woods Hole*, **90**, 122.
Morse, H. N. (1902). *Z. phys. Chem.* **41**, 733.
Sherrill, M. S. (1903). *Z. phys. Chem.* **43**, 734.
Sherrill, M. S. (1904). *Z. phys. Chem.* **47**, 103.
von Stackelberg, M. & von Freyhold, H. (1940). *Z. Elektrochem.* **46**, 120.
Thomas, H. C. (1939). *J. Amer. Chem. Soc.* **61**, 920.

THE SENSITIVITY OF BARNACLES AND THEIR LARVAE TO COPPER AND MERCURY

By K. A. PYEFINCH and JOAN C. MOTT
Biologists, The Corrosion Committee, British Iron and Steel Research Association, from the Marine Station, Millport

(*Received* 15 *November* 1947)

(With Six Text-figures)

INTRODUCTION

A number of workers (e.g. Powers (1917), Löhner (1924), Carpenter (1927, 1930), Ludwig (1927), Hykes (1931), Jones (1935, 1937, 1938, 1939, 1941, 1947), Miller (1946)) have investigated the effects of heavy metal poisons on a variety of animals, including Protozoa, Ctenophores, Platyhelminthes, Crustacea, Molluscs, Polyzoa and Teleosts. Cole (1932) published an account of the effect of a number of substances, some poisonous, on the rate of cirral beat of barnacles and, more recently, Müller (1940) and Clarke (1947) have added further to our knowledge of the toxic effects of copper and mercury on larval and adult barnacles.

Clarke's investigation (1947) was carried out in connexion with an extensive investigation of the fouling problem, and the experiments described below also formed part of a fundamental study of the same problem. In the present instance experimental work has chiefly been carried out using the larvae and adults of *Balanus balanoides*, though some observations have been made on the corresponding stages of *B. crenatus*. As sufficient numbers of the larvae of both these species are only present in the plankton for short periods each year (Pyefinch, 1948*b*), experimental work involving these stages has been virtually limited to the month of April and this study has, therefore, been spread over some four years in all. It will be clear that much more information is needed before even the outline of this investigation can be regarded as complete, but this preliminary study is presented as a further contribution to what is already known about the effects of poisons on the acorn barnacle, undoubtedly the most important type of animal fouling organism.

GENERAL EXPERIMENTAL TECHNIQUE

'Analar' cupric sulphate ($CuSO_45H_2O$) and mercuric chloride ($HgCl_2$) were used as sources of copper and mercury respectively. Stock solutions of these salts were made up in distilled water and the appropriate volume of the stock solution added to freshly filtered sea water for each experiment. The use of salts such as cupric sulphate severely limited the range of concentrations which could be used in natural sea water as, if the concentration of copper rises above 6–7 mg./l., precipitation of basic copper salts occurs.

Some experiments using this poison were therefore carried out in an artificial sea water of the following composition:

	g.		g.
NaCl	23·48	$CaCl_2$	1·10
$MgCl_2$	4·98	KCl	0·66
Na_2SO_4	3·92		

Distilled water to 1000 g. The solution was well aerated before use.

It is realized that the copper concentrations in natural sea water just quoted far exceed the true solubility of this element in this medium. The solution of copper in sea water is a complex problem, which is more fully discussed by Barnes & Stanbury (1948), and all that can be added here is that, up to concentrations of 6–7 mg. Cu/l., an increase in copper concentration in natural sea water produces an increased toxic effect. The means whereby this is produced are at present obscure.

No complications of this character occurred with mercuric chloride, and this reagent could be used in natural sea water over the whole range of concentrations employed in these experiments.

The concentration of poison present was determined either during or immediately after each experiment, using colorimetric methods. For the estimation of copper, the sodium diethyl-dithio-carbamate method was used (for details see Harris (1947); both the direct and the extraction techniques described in this paper were employed) and the amount of mercury present was estimated using the diphenyl-thiocarbazone (dithizone) technique (see Barnes, 1946; Harris, 1947). The transmittancies of the solutions were determined, by means of a Spekker Photoelectric Absorptiometer, fitted with Ilford spectrum violet filters (no. 601) for the copper determinations and with Ilford spectrum blue-green filters (no. 603) for the mercury determinations. In general, agreement between calculated and determined concentration was good.

For each experiment, the range of concentrations used was such as to cover the whole range between no apparent lethal effect and 100% lethal effect at the end of the exposure period which, with the exception of cases specifically noted, lasted 6 hr. The percentage killed at each concentration was plotted, and the median lethal concentration determined from this graph.

Further details of experimental technique are given in the later sections of this paper.

TOXIC EFFECTS OF COPPER AND MERCURY ON NAUPLII

A small number of experiments was carried out on the effects of copper and of mercury on the later stage nauplii of *B. balanoides* and *B. crenatus*. These experiments were made merely to obtain an estimate of the sensitivity of these larval stages, for comparison with that of the cyprid and the adult barnacle; an extensive survey of the effects of these poisons was not considered likely to be profitable.

In these experiments an aliquot portion of a suspension of the nauplii of the two species was added to the poison solution in small crystallizing dishes, so that the final volume of the solution was 50 ml. At the beginning of the exposure period in each concentration used the nauplii swam freely, and quickly took up a position

either immediately below the meniscus on the side of the dish towards the light or at the bottom angle of the dish on the side away from the light. (These are the normal reactions of these larvae. Ishida (1936) has described similar responses for the nauplii of *B. amphitrite albicostatus* and *Tetraclita squamosa* and has termed them 'skotophobic' and 'photophobic' respectively; in the present instance it would seem that both responses could be due to a positive reaction to light, since a marked 'lens' effect is present at the bottom angle of a cylindrical glass vessel.) Shortly before the end of the 6 hr. exposure period each dish was rotated through 90°; those larvae still capable of active movement took up their orientation afresh to the incident light; those moribund or dead remained in their old positions. At the end of the exposure period it was thus possible to remove the active and the inactive or moribund larvae separately. They were fixed in dilute formalin immediately after removal, and the numbers counted and the stages analysed (following the methods described elsewhere (Pyefinch, 1948*a*)).

The result of one experiment is shown in Table 1.

Table 1. *Toxic effect of copper and mercury on the nauplii of* Balanus balanoides *and* B. crenatus

Stage or stages	Median lethal concentration (mg./l.)		Copper/mercury (wt./wt.)
	Copper	Mercury	
B. crenatus IV	0·27	0·06	4·5/1
B. crenatus V	0·23	0·08	2·9/1
B. crenatus VI	0·26	0·16	1·6/1
B. crenatus, all stages	0·26	0·09	2·9/1
B. balanoides III	0·34	0·09	3·8/1
B. balanoides IV	?0·38	0·23	1·7/1
B. balanoides V	0·46	0·30	1·5/1
B. balanoides VI	0·46	0·30	1·5/1
B. balanoides, all stages	0·41	0·23	1·8/1

In each stage, mercury is more poisonous than copper; it is of interest to note that, although the sensitivity of the nauplii of both species to copper does not change considerably as one larval stage succeeds another, there is a more marked change in the sensitivity to mercury. The ratio between the median lethal concentrations of the two poisons, therefore, decreases through the series.

These results cannot be compared directly with those of Clarke (1947) for the nauplii of *Balanus eburneus*, as, since his tests were made on the day the nauplii were released from the brood pouch, it is probable that only first and second stage nauplii were present, and also his experiments were continued for periods of 22, 29 and 48 hr. However, from the data he gives (Clarke, 1947, table II), it is possible to estimate the median lethal concentration for the 22 hr. exposure period; this value is roughly 0·15 mg. Cu/l., which suggests that the sensitivity of the nauplii of *B. eburneus* is of the same order as that of the nauplii of *B. balanoides* and *B. crenatus*.

TOXIC EFFECTS OF COPPER AND MERCURY ON FREE-SWIMMING CYPRIDS

The experiments with free-swimming cyprids, which formed the major part of this investigation, were carried out in small glass bottles, each of approximately 130 ml. capacity. Duplicate determinations were usually made at each concentration of poison. Ten cyprids were placed in each bottle and, after the addition of the larvae, the bottle was filled to the brim and capped with a glass plate. The latter procedure was adopted because it was anticipated that the cyprids would become trapped in the surface film; later it was found that this possibility was only likely to occur with the cyprid of *B. crenatus*, and as most of the experimental work with this stage was carried out with *B. balanoides* this precaution was not really necessary. The rate of oxygen consumption of the cyprid, however, is very low (of the order of 0·0007 ml. O_2/day)* so that oxygenation of the test solutions was probably completely satisfactory, although the surface was sealed from contact with the air.

The cyprid, both of *B. balanoides* and of *B. crenatus*, is by no means continuously active, and a number of different methods were tried as indicators of activity at the end of the exposure period. That finally adopted was as follows. At the end of the experiment, the cyprids were removed from the bottle with a pipette and placed in a Petri dish. As much as possible of the poison solution carried over in this process was removed and the dish filled with fresh filtered sea water. The dish was then placed on a sheet of white paper marked with ten dots; each cyprid was brought into position over one of the dots and those which remained in this position over a period of at least 12 hr. after the conclusion of the experiment were counted as dead. Usually several inspections were made during this 12 hr. period.

The results of a number of determinations (made in 1947) show that the average median lethal concentration for the cyprid of *B. balanoides* is 5·9 mg. Cu/l., whereas the corresponding value for mercury (based on a smaller number of determinations) is 3·0 mg./l., giving a copper/mercury ratio of almost 2/1 (weight/weight). The free-swimming cyprid of *B. crenatus* differs markedly in its sensitivity to these two poisons; only a small and variable fraction of the cyprids is killed in the concentrations of copper possible in natural sea water, so it is reasonable to assume that the median lethal concentration for this poison is greater than 7·0 mg./l., but it is much more sensitive to mercury than the cyprid of *B. balanoides*, as the median lethal concentration is appreciably less than 1·0 mg./l. For this cyprid, the copper/mercury ratio might well be of the order of 10/1.

In carrying out estimations of the sensitivity of cyprids of *B. balanoides*, it is essential to use comparable material. For these experiments, large numbers of the cyprids of this species were collected from the plankton at the time when they are present in considerable numbers, and stored in tanks in the laboratory. Settlement of the cyprids did not take place immediately, but usually occurred 4 or 5 days after catching. During this period the median lethal concentration, both for copper and for mercury, decreases; examples of the amount of this decrease for copper are given in Table 2.

* We are indebted to Mr W. R. Hunter for this observation.

Comparable decreases occur in the median lethal concentration of mercury so that, by selection of experimental results, it is possible to produce wide variations in the relative toxic effect of copper and mercury. Table 3 gives examples of some ratios that can be obtained.

Table 2. *Decrease in median lethal concentration of copper during laboratory storage*

Period in storage tanks (days)	Median lethal concentration (mg. Cu/l.)
1	5·9
4	2·1
1	2·5
5	1·2

Table 3. *Median lethal concentrations after different periods of storage*

Period in storage tanks (days)		Median lethal concentration (mg./l.)		Cu/Hg
For copper determination	For mercury determination	Copper	Mercury	
1	1	4·4	2·7	1·6/1
4	1	2·4	2·7	0·9/1
1	11	3·7	0·75	4·1/1
10	11	1·8	0·75	2·4/1

The experiments which formed the basis of Table 3 were carried out in 1945, and it will be noted that, where comparable material (i.e. material which had been stored for the same length of time) was used, the copper/mercury ratio approximated to the value indicated by more extensive work 2 years later.

It seemed possible that these changes in sensitivity to these poisons (and particularly the change in sensitivity to copper, since there is a good deal of evidence which suggests that this element is closely bound up with intracellular processes and especially with intracellular respiration) might be an indication of physiological changes taking place during the life period of the cyprid. This suggestion received some support, on the basis of experiments made during 1945, from the variation in sensitivity to copper from day to day. These results are shown in Fig. 1. The median lethal concentration varies from day to day, and shows some correlation with the variation in numbers of larvae taken in the plankton each day and also, though this is not shown in the figure, with the rapidity with which these larvae settled under laboratory conditions. For example, the larval stocks

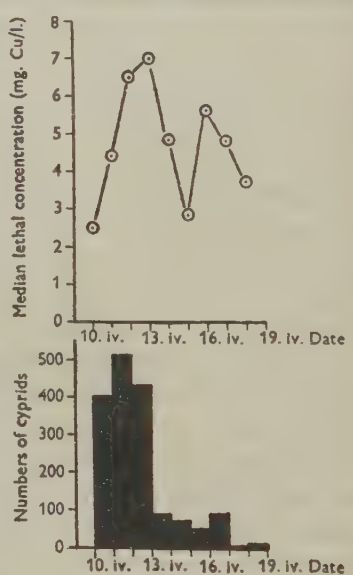


Fig. 1. *Balanus balanoides*. Lethal effect of copper on daily batches of cyprid larvae and numbers of cyprids caught day by day, April 1945.

from which samples were drawn for the sensitivity determinations made on 14 and 15 April began to settle in a time much shorter than the 4 or 5 days suggested as an average interval earlier in this account; the next day the sensitivity decreased, the numbers of larvae caught increased and the interval between catching and settlement also increased. One interpretation of this sequence of events is that the tests on 14 and 15 April were made on larvae which were approaching the end of their life period, whereas the test on the following day was made on cyprids which had recently moulted from the sixth stage nauplius.

This hypothesis was further tested by more extensive experiments carried out through April 1947; their results are shown in Fig. 2. This shows that the corre-

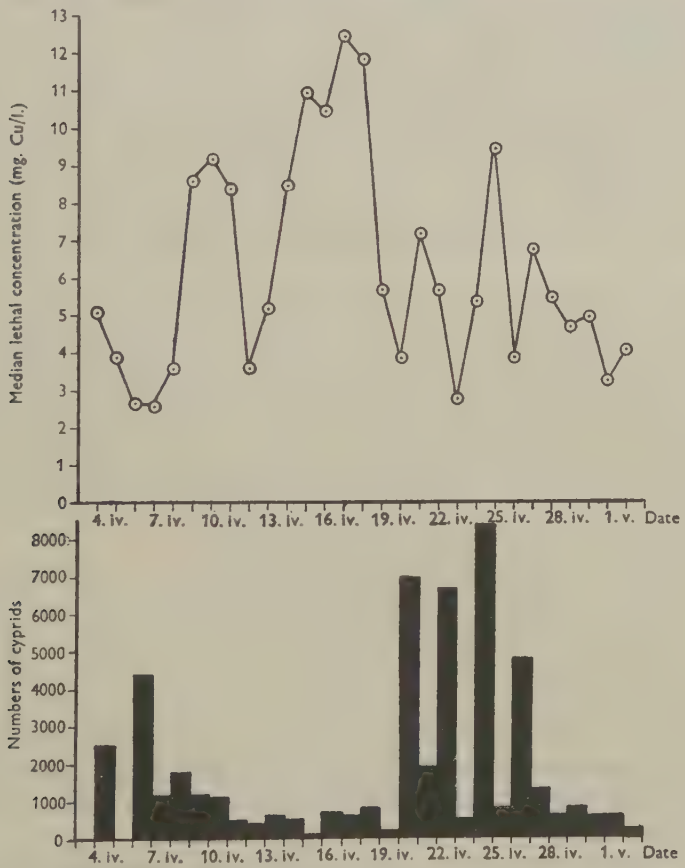


Fig. 2. *Balanus balanoides*. Lethal effect of copper on daily batches of cyprid larvae and numbers of cyprids caught day by day, April 1947.

lation between a decrease in sensitivity and an increase in numbers of cyprids taken in plankton hauls suggested by the determinations made in 1945 applies only to the closing period, and possibly also to a short period at the beginning of the 1947 experiments. A full correlation throughout the period of cyprid abundance in 1947 was not to be expected, as it is evident from Fig. 2 that the numbers of larvae caught

day by day fluctuated considerably for much of the period over which sensitivity determinations were made. This suggests that the cyprid populations were, for some time, made up of a series of small batches of newly formed individuals. Unfortunately, there is no possibility, at present, of testing such a hypothesis by direct observation. Visscher (1928) found that the oil globules present in the tissues of the anterior end of the cyprids of *B. amphitrite* and *B. improvisus* disappeared as the time for settlement approached, but this change does not occur in the cyprid of *B. balanoides*. However, the relationship between sensitivity to copper, numbers of cyprids present in the plankton and the ease with which settlement occurred under laboratory conditions found in 1945, and confirmed over the later part of the readings 2 years later, suggests that variations in sensitivity to copper may be a valid indication of changes in physiological state.

It should be added that the relationship observed in 1945 is perhaps unusually clear, as daily sensitivity determinations were only made over the latter part of the period of cyprid abundance. Had these determinations been begun earlier, it is possible that the indications would have been less clear, because the cyprid stock earlier in the period of abundance of these larvae would have been composed, as it was in 1947, of a mixture of cyprids of all ages, some newly hatched and some ready to settle.

B. crenatus cyprids settle much more readily under laboratory conditions than the cyprids of *B. balanoides* and, so far as can be judged on the basis of a few experiments, there are no changes in the sensitivity of the cyprids of this species on storage comparable with those found for *B. balanoides*. This difference may merely be due to the fact that the conditions of laboratory storage are more natural for the cyprids of *B. crenatus* than for those of *B. balanoides*, since physiological changes in the latter must, it may be presumed, be markedly affected by the alternate immersion and exposure these cyprids encounter between tide marks.

Some estimations of the sensitivity of *B. balanoides* cyprids have been made in artificial sea water. The use of this medium enables a wider range of copper concentrations to be used and the results of the exposure of the cyprids of *B. balanoides* to such a range of concentrations in this medium are shown in Fig. 3. Lethal action increases with concentration up to 0·5 mg. Cu/l., but, from concentrations of this order up to 100 mg. Cu/l., lethal action decreases as the concentration increases, so that at the latter concentration, 90% of the cyprids used recover when returned to sea water after a 6 hr. exposure period. If the concentration is increased further, the lethal effect again increases.

These results may be due to the use of artificial sea water, and thus cannot be compared with exposures using natural sea water. But the use of artificial sea water does not produce any striking differences in the action of mercury, and other (unpublished) work suggests that results obtained in artificial sea water are comparable with those in the natural medium. If these results are therefore truly due to the poison, and not to the medium, they suggest an interesting difference in the mode of action of copper and of mercury. It is not possible to elucidate the nature of this difference on the basis of the present series of experiments, but one possible

explanation of the results obtained from exposures to copper in artificial sea water is that this element not only has a direct poisoning action which may be comparable with that of mercury, but also has an effect on some metabolic process which affects the rate of uptake of the poison. It should be added that, when tests are carried out in natural sea water, if the stock used is more than usually sensitive, the lethal effect of the highest copper concentrations possible in this medium is not as great as that of lower concentrations, an observation which agrees with the results obtained in artificial sea water and which perhaps lends further support to the thesis that the latter may be regarded as typical.

A number of experiments has been made in which cyprids of *B. balanoides* were exposed to mixtures of copper and mercury; in some of these the concentration of copper was held constant and that of mercury varied, in others varying amounts of copper were added to constant amounts of mercury. In yet other experiments the

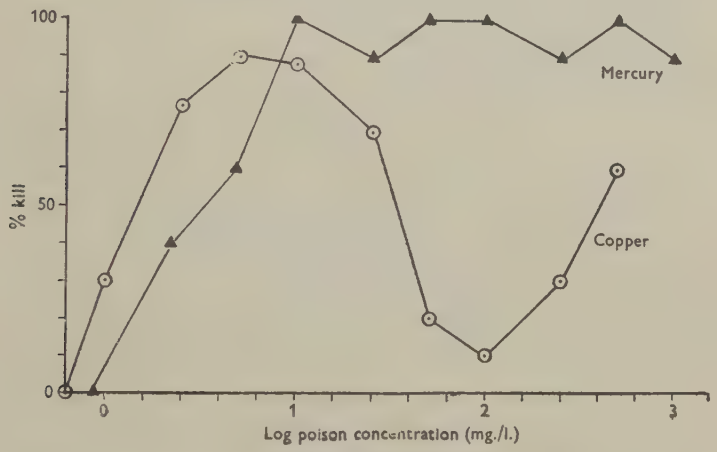


Fig. 3. Lethal effect of copper and of mercury, in an artificial sea water, on the cyprid larvae of *Balanus balanoides*.

cyprids were first exposed to one poison and then transferred to solutions containing the other.

These preliminary experiments show clearly that the effect of a mixture of these two poisons is complex, and that their combined effect is not simply additive, even if allowance is made for the greater toxic effect of mercury. Fig. 4 compares the effect of copper alone, mercury alone and of mixtures of the two poisons on the survival time, and Fig. 5 the effect of increasing concentrations of the mixed poisons for a constant exposure period of 6 hr., together with typical curves for the effect of each poison alone under similar conditions. Fig. 5 indicates that mixtures of copper and mercury are more toxic than either alone at lower concentrations, but that this relationship does not hold at higher concentrations.

Other experiments have been made using the cyprids of *B. balanoides* in an attempt to discover some difference in the mode of action or method of entry of copper and mercury. Though their results do not form part of the main theme of

this paper, it is felt that they deserve mention as examples of the effect of other factors on heavy metal poisoning.

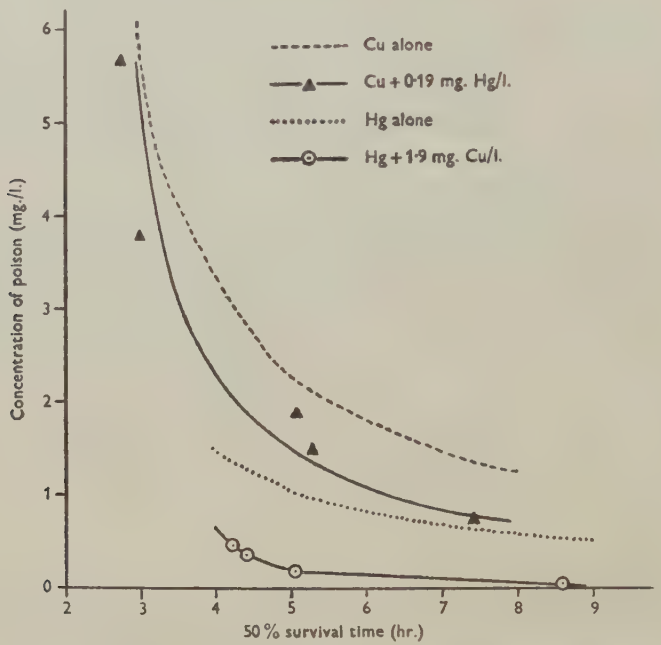


Fig. 4. Survival of the cyprid larvae of *Balanus balanoides* in copper solutions, in mercury solutions and in solutions containing both these poisons.

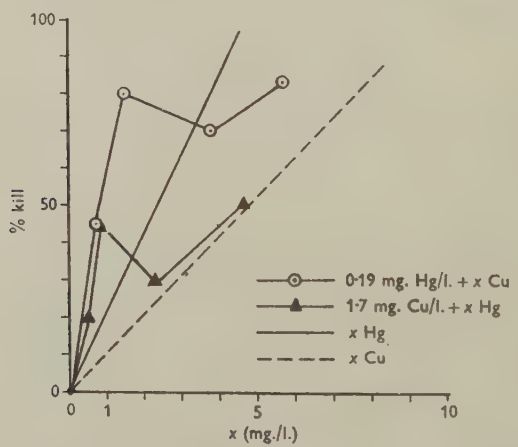


Fig. 5. Lethal effect of solutions containing copper and mercury on the cyprid larvae of *Balanus balanoides*.

Cyprids of *B. balanoides* can survive for considerable periods in diluted sea water, and only small numbers (of the order of 20%) are killed by exposure for 24 hr. to fresh water. If exposed to the action of copper or of mercury in diluted sea water,

the toxicity of both these poisons is markedly reduced. Tables 4 and 5 indicate the extent of this reduction.

Table 4. *The effect of copper in diluted sea water*

Solution used	Median lethal concentration (mg./l.)	Age* of larvae (days)
Sea water	2·45	1
75 % sea water + 25 % tap water	6·7	1
50 % sea water + 50 % tap water	7·0	1
Sea water	1·8	10
75 % sea water + 25 % distilled water	2·1	10
50 % sea water + 50 % distilled water	?7·5	10

* Period in storage tanks.

Table 5. *The effect of mercury in diluted sea water*

Solution used	Median lethal concentration (mg./l.)	Age* of larvae (days)
Sea water	2·7	1
75 % sea water + 25 % tap water	2·0	1
50 % sea water + 50 % tap water	4·7	1
Sea water	0·75	11
75 % sea water + 25 % distilled water	0·90	12
50 % sea water + 50 % distilled water	2·1	11

* Period in storage tanks.

These results also have a practical significance, since they must mean that copper and mercury leaching from an anti-fouling paint in estuarine conditions will be much less effective. As the leaching rate of these poisons is itself reduced in water less saline than sea water, it would seem that an anti-fouling composition, from which copper and mercury can be leached at an adequate rate in sea water, could be completely ineffective in the estuarine conditions prevailing in some harbours.

Hypertonic sea water (prepared by slow evaporation of natural sea water) also seems to have no effect on cyprids of *B. balanoides*, but the toxic effect of copper is markedly reduced in this medium. Some of the results are shown in Table 6.

Table 6. *The effect of copper in hypertonic sea water*

Sea water	Specific gravity	Median lethal concentration (mg./l.)	Age* of larvae (days)
Normal	1024	0·8	15
Hypertonic	1031	3·4	15
Normal	1027	1·3	24
Hypertonic	1047	7·0	24

* Period in storage tanks.

As this reduction in toxicity, at least for copper, occurs both in hypo- and hypertonic sea water, it would seem unlikely that this phenomenon can be explained

in terms of a direct osmotic effect, since that should mean that the larvae, if they were less sensitive in hypotonic, should be more sensitive in hypertonic solutions. A characteristic feature of the larvae immersed in hypo- and hypertonic sea water is their immobility—the antennae and thoracic appendages are withdrawn within the carapace and the larva rests on its side. Possibly this lack of movement is bound up with their decreased sensitivity to poisons.

It is possible that the rate of penetration of poisons is governed by the state of the bounding integument, and if this state is altered, e.g. by carrying out exposures to copper and to mercury in the presence of soaps, different rates of penetration and differences in toxic effect might occur. In carrying out experiments along these lines it was hoped that differences might be revealed in the rate of penetration of copper and of mercury. Unfortunately, as the results in Table 7 show, neither hope was fulfilled, the toxic effect did not vary with the soap concentration, and there was no significant difference between the effect of copper and of mercury with or without sodium oleate.

Table 7. *The effect of copper and mercury in the presence of sodium oleate*

Poison present	Median lethal concentration (mg./l.) of poison in the presence of sodium oleate at concentrations of (mg./l.)					
	0	$1{\cdot}25 \times 10^{-4}$	$1{\cdot}25 \times 10^{-3}$	$1{\cdot}25 \times 10^{-2}$	$1{\cdot}25 \times 10^{-1}$	1·25
Copper	2·2	1·9	2·2	2·1	2·3	2·7
Mercury	0·5	0·45	0·65	0·6	0·6	—

The two sets of estimations were not carried out on larvae of equivalent age, so that the copper/mercury ratio is not typical.

EFFECT OF COPPER AND MERCURY ON SETTLEMENT

Two methods have been used to investigate the effect of exposure to copper and to mercury on the settlement of *B. balanoides* cyprids. At first, batches of cyprids were placed in low concentrations of the poisons in breffits, the total volume of solution was approximately 2 l., and observations on activity and the occurrence of settlement made at intervals. In this series of experiments the cyprids remained in the poison solution throughout. In a later series of experiments the period of exposure to the poison was limited and, after the end of the exposure period, the cyprids were washed with clean filtered sea water and then transferred to a further quantity of filtered sea water for observations of activity and settlement, which, as in the first set of experiments, were continued for some days. Each method has its disadvantages: in the first the poison concentration decreases over the period of observation (probably due to adsorption), and in the second the cyprids are finally transferred to a clean container which almost certainly does not present the optimum conditions for settlement.

In all cases, active cyprids were used from laboratory stocks in which appreciable settlement was taking place. If the cyprid of *B. balanoides* has to reach a certain

physiological state before settlement is possible, it may therefore be assumed that a reasonable proportion of the stock used had reached that state.

The results of experiments carried out according to the first method, in which the larvae were exposed to low concentrations of poison over considerable periods, are set out in Tables 8 and 9.

Table 8. *The effect of copper on settlement*

Concentration of copper (mg./l.)	Period cyprids remained active (days)	Number settled	Percentage settlement
	Exp. 1. Duration, 8 days		
Control	8	137	68
0·024	8	1	1
0·06	5	0	0
0·1	3	0	0
0·5	1	0	0
	Exp. 2. Duration, 7 days		
Control	7	191	81
0·01	7	2	1
0·03	4	0	0
0·08	3	0	0
0·45	1	0	0

Table 9. *The effect of mercury on settlement*

Concentration of mercury (mg./l.)	Period cyprids remained active (days)	Number settled	Percentage settlement
Control	19	1100	50
0·01	19	440	38
0·05	10	0	0
0·1	10	0	0
0·2	2	0	0

Duration of experiment, 19 days.

The results of these experiments suggest that extremely low concentrations of both these poisons can prevent settlement, which agrees with the increasing sensitivity to copper during laboratory storage which was noted in the previous section (p. 280), and they also suggest that very low concentrations of copper are more effective in this respect than comparable concentrations of mercury. Though the latter observation is supported by observations on nauplii and free-swimming cyprids, where again the lowest concentrations of mercury used were less effective than the lowest concentrations of copper (though this relationship is reversed before concentrations are reached which are high enough to kill 50 % of the larvae), another explanation is possible. Concentrations of these poisons of the order used in these experiments are by no means stable, and the difference between the lowest copper and mercury solutions may merely be due to the more rapid disappearance (by adsorption on to the walls of the vessel or on to organic matter in suspension) of the latter element. Mercury certainly does disappear quickly as, in a later experiment

on these lines, solutions which contained 0·019 mg./l. and 0·08 mg./l. at the beginning of the experiment contained no detectable mercury 4 days later.

Tables 10 and 11 give the results obtained using the second method, in which the period of poison exposure was limited and the further history of the larvae followed in fresh sea water.

Table 10. *Settlement after exposure to copper for varying periods*

Concentration of copper (mg./l.)	Exposure periods (hr.)								
	0·25	0·50	1·0	2·0	3·0	6·0	12·0	24·0	48·0
0·1	—	—	—	—	—	XXXX Good	XXXX NS	XX NS	XX NS
0·25	—	—	—	—	—	XXX Trace	XXX NS	X NS	X NS
0·50	—	—	—	—	—	XXX Trace	XX NS	—	—
1·0	—	—	XX NS	XX NS	XX NS	XXX Trace	—	—	—
2·5	—	XXX NS	X NS	O NS	—	O NS	—	—	—
5·0	XXXX NS	X NS	X NS	O NS	—	O NS	—	—	—

Table 11. *Settlement after exposure to mercury for varying periods*

Concentration of mercury (mg./l.)	Exposure periods (hr.)								
	0·25	0·50	1·0	2·0	3·0	6·0	12·0	24·0	48·0
0·01	—	—	—	—	—	XXXX Trace	XXX NS	XXX Light	XXX NS
0·05	—	—	—	—	—	XXXX NS	XX NS	XXX NS	XX NS
0·14	—	—	—	—	—	XXX NS	XX NS	X NS	—
0·57	—	—	—	—	XXX NS	XX NS	XX ? Trace	—	—
1·28	—	—	XX ? Trace	—	XXX Light	XX Trace	—	—	—
3·3	XX Trace	XX Trace	X NS	XX Trace	—	—	—	—	—

These tables record estimates of the activity of the cyprids at the end of the poisoning period (XXXX, moderately active; XXX, some active; XX, a few active; X, isolated individuals active; O, no activity) and estimates of the amount of settlement (Good, Some, Light, Trace and No Settlement (NS)). A dash indicates no exposure for that period and concentration.

The results of short-period exposures to copper (Table 10) indicate that settlement is only possible after comparatively short periods of exposure to the lowest concentrations used and further that, though many or most of the cyprids may be

active at the end of the exposure period, those individuals surviving in an active state are not capable of settlement. The results for mercury (Table 11) present rather a different picture. The concentrations of this poison used in these experiments had generally less effect on activity, but settlement subsequently took place mainly in stocks which had been exposed to higher concentrations of mercury.

The results for copper further confirm the inhibitory effect of very low concentrations of this poison on settlement, whereas those for mercury suggest something of a stimulatory effect for the higher concentrations used. The results of neither set of experiments can be regarded as wholly satisfactory, and it would be unwise to speculate further on results obtained under such artificial conditions.

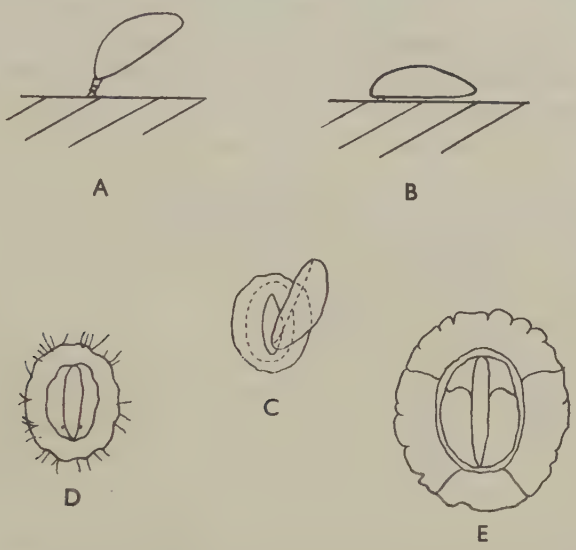


Fig. 6. Stages in the metamorphosis of the cyprid larva of *Balanus balanoides*.

EFFECT OF COPPER ON METAMORPHOSING CYPRIDS

The experiments on the sensitivity of the cyprid of *B. balanoides* during storage in the laboratory, and on the effect of copper on settlement, suggest that this larva becomes very sensitive to copper at the time of attachment. A series of tests was therefore made on cyprids of this species which had settled and which were metamorphosing into the adult form. The morphological changes which occur during this process were not followed in detail, but it was found possible to recognize five stages in the sequence (Fig. 6) which may be described as follows.

Stage A. Immediately after attachment; the body of the cyprid projects upwards from, and makes an acute angle with, the substratum. The antennae are firmly anchored and permanent attachment has been achieved. This stage is probably short-lived, but it lasts long enough to be recognized in an appreciable proportion of a population of recently attached cyprids.

Stage B. The body of the cyprid now lies close to the substratum, the anterior

end is appreciably flattened and the tissues internally show a distinct median grove anteriorly.

Stage C. The major external changes are now complete, and the form of the adult has been assumed. Shedding of the cyprid integument is not complete, as the thoracic appendages still remain within their original integument. The cyprid carapace, therefore, remains attached to the young barnacle and seems to remain so for some little time.

Stage D. The cyprid carapace has now been shed completely. Calcification has not yet begun and the perimeter of the basis bears scattered tufts of fine setae.

Stage E. Calcification has begun and the outlines of the primary compartments are clear. The paired eyes, clearly visible until this stage, are no longer apparent.

Small populations of *B. balanoides* cyprids which had settled, mainly on *Mytilus* shells, during the 24 hr. immediately preceding the experiment, were placed in a series of copper solutions and observations made on their further progress. All the individuals used were initially in Stage B.

The results of two experiments are shown in Table 12.

Table 12. *The effect of copper on metamorphosing cyprids of* Balanus balanoides

Period of exposure to copper (hr.)	Copper concentration (mg./l.)		Numbers of cyprids	Numbers of cyprids at stages			
	Nominal	Measured		B	C	D	E
39	Control		15	0	0	0	15
	0·01	0·003	17*	0	0	17	0
	0·05	0·029	16*	0	0	16	0
	0·1	0·09	19	1	1	17	0
	0·5	0·46	18	0	1	17	0
	1·0	0·90	19	0	0	19	0
33·5	1·0	—	5	0	0	5	0
	2·5	—	4	0	0	4	0
	5·0	—	12	0	1	11	0
	7·5	—	8	4	0	4	0
	10·0	—	6	0	0	6	0
	Control		3	0	0	0	3

* Calcification began when transferred to fresh sea water.

The results given in this table show clearly that the sensitivity during metamorphosis is far lower than either that of the cyprid just before settlement or (as will be shown in the following section) of the barnacle after metamorphosis. Metamorphosis can continue in all the concentrations of copper possible in natural sea water, though it should be noted that, as calcification only begins when individuals which have metamorphosed in the two lowest concentrations used are transferred to fresh sea water, death occurs in the remaining cases immediately after this process has been completed.

The low sensitivity of the cyprid during metamorphosis is probably the explanation of a phenomenon frequently noticed in raft exposures of anti-fouling compositions—that settlement and metamorphosis can take place on a surface too toxic for

the continued life of the barnacle after metamorphosis. Individuals settling under such circumstances do not become calcified and die within a few days after metamorphosis.

Experiments have also been carried out on the sensitivity of the cyprid of *Balanus crenatus* during metamorphosis. The main external changes during the metamorphosis of this species are generally the same as those of *B. balanoides*, with two exceptions:

(*a*) Stage C is virtually absent, as the cyprid carapace is rapidly shed at the conclusion of metamorphosis. (This stage is therefore omitted from the tables below.)

(*b*) Calcification does not begin so soon after the completion of metamorphosis. Further progress is, however, marked by the appearance of the compartments and of the outlines of the scuta and terga. The stage shown as D′ in the tables below refers to individuals which have reached this condition.

The results of these experiments are summarized in Tables 13 and 14. All the cyprids used were initially in Stage B.

Table 13. *The effect of copper on metamorphosing cyprids of* Balanus crenatus

Treatment	Copper concentration (mg./l.)		Numbers of cyprids	Numbers of cyprids at stages		
	Nominal	Measured		B	D	D′
After 10 hr. exposure to copper	0	—	9	1	8	
	0·5	—	13	3	10	
	1·0	—	21	2	19	
	2·5	—	14	4	10	
	5·0	—	12	4	8	
	7·5	—	13	1	12	
After 24 hr. exposure to copper	0	0	9		9	
	0·5	0·42	13		13	
	1·0	0·72	21		21	
	2·5	1·7	14		14	
	5·0	2·6	12	1	11	
	7·5	5·5	13		13	
After further 32 hr. in fresh sea water	0	—	9			9
	0·5	—	13		13	
	1·0	—	21		21	
	2·5	—	14		14	
	5·0	—	12		10	
	7·5	—	13		13	

These results show clearly that the cyprids of *B. crenatus* are able to complete their metamorphosis in all the concentrations of copper used but, judging by their failure to progress beyond Stage D (except for the controls), they can be killed by low concentrations of copper immediately metamorphosis has been completed.

The measurements of the copper content of the solutions (and for the mercury solutions given in Table 14) were made at the end of the exposure period. It is most unlikely that the initial concentrations of the poison solutions were below the nominal values indicated, so that the difference between the minimal and measured values gives a rough guide to the range of concentrations existing in these solutions during the test period.

Table 14. *The effect of mercury on metamorphosing cyprids of* Balanus crenatus

Treatment	Mercury concentration (mg./l.)		Numbers of cyprids	Numbers of cyprids at stages		
	Nominal	Measured		B	D	D′
After 10 hr. exposure to mercury	0	—	4	1	3	
	0·5	—	11	8	3	
	1·0	—	23	10	13	
	2·5	—	15	4	11	
	5·0	—	18	15	3	
	7·5	—	14	7	7	
	10·0	—	24	17	7	
After 24 hr. exposure to mercury	0	0	4		4	
	0·5	0·27	11	6	5	
	1·0	0·41	23	9	14	
	2·5	1·5	15	4	11	
	5·0	5·1	18	14	3	
	7·5	6·7	14	7	7	
	10·0	12·3	24	17	7	
After further 32 hr. in fresh sea water	0	—	4			4
	0·5	—	11	6	5	
	1·0	—	23	9	14	
	2·5	—	15	4	11	
	5·0	—	18	14	3	
	7·5	—	14	7	7	
	10·0	—	24	17	7	

The results with mercury are not so clearly defined as those obtained using copper. Some metamorphosis appears to have been possible in all the concentrations of mercury used, but its extent bears little relationship to the concentration. Further, if the state after 10 hr. exposure is compared with that after 24 hr. exposure it is seen to be the same, with the exception of the 0·5 and 1·0 mg./l. solutions, in both cases. A possible interpretation of these results is therefore that, above a concentration of 1·0 mg./l. (or less), mercury is toxic, but that it takes a little time to exert its effects. Thus, in a population of metamorphosing cyprids, in which some have recently entered Stage B and some are much further advanced, the latter may be able to complete their metamorphosis before sufficient poison has been absorbed to stop the process.

Thus it would appear that the cyprid of *B. crenatus* is as insensitive to copper during metamorphosis as that of *B. balanoides*, and it may be suggested that the former species is much more sensitive to mercury. It is of interest to note that the sensitivities of the metamorphosing cyprid of *B. crenatus* are similar to those of the free-swimming larva, whereas the sensitivity of the metamorphosing cyprid of *B. balanoides* is very different (at least for copper) from that of the free-swimming larva immediately before settlement.

THE EFFECT OF COPPER AND OF MERCURY ON BARNACLES

This series of studies on the sensitivity of the larval stages of *B. balanoides* and *B. crenatus* was completed by a series of estimations of the sensitivity of barnacles after metamorphosis.

For this purpose, individuals which had settled on ground glass microscope slides were used. Before each experiment, the slides used were cleaned as thoroughly as possible (to remove as much organic matter as was practicable, so as to present the minimal surface for adsorption of the heavy metal ions) and then placed in the poison solutions. Each container had a capacity of approximately 175 ml., and a slow circulation (4–5 l./24 hr.) of the poison solution was maintained through the containers for the whole of the exposure period. Normally the latter was 6 hr. (so that the results of these experiments are comparable in this respect with those on free-swimming cyprids) but in a few experiments it was 24 hr. in length.

Immediately after the end of the experiment, the slides were removed from their containers, washed in running sea water and placed under sea-water circulation. Each was then examined and the activity of each individual recorded according to the following scheme:

Active. Full and rapid movements of the cirri possible.

Moderately active. Some cirral movement possible, but cirri never fully extended, frequency of movement may approach normal.

Slow movement. If the opercula are closed, they respond to touch; if the opercula are open and the cirri are protruding the latter can execute slow (often spasmodic) movements or can be stimulated to do so with a needle.

Inactive. No response of closed opercula to touch and no response of cirri, if protruding.

The slides were then reimmersed in the sea and again examined a week or 10 days later. After this interval, those which had been killed during the exposure to poison or which had died later were immediately obvious, as their shells were empty, whereas those which had survived or recovered were fully active.

Tables 15 and 16 show the results of two typical experiments in which *B. crenatus* was exposed, in one case to copper and in the other to mercury.

These tables not only illustrate the way in which the results of these tests were assessed, they also show something of the characteristics of copper and mercury poisoning of barnacles. It will be noted that the 'Apparent Survival' is a more reliable guide to ultimate survival when copper has been used as a poison than is the case when the barnacles have been exposed to mercury, which suggests that copper is more immediate (and possibly less permanent) in its effects than mercury.

Comparisons of the sensitivity of *B. balanoides* and *B. crenatus* to these two poisons reveal remarkable differences. Table 17 shows the results of determinations made on these two species over the period following metamorphosis. Those on the left-hand side of the table for each species are the results obtained for the youngest specimens tested; those on the right-hand side of the table those for the oldest specimens tested.

The experiments on *B. balanoides* cover a period of about $2\frac{1}{2}$ months from settlement, those on *B. crenatus* a period of just over 3 months from settlement.

Copper and mercury seem roughly equi-toxic (weight for weight) to *B. balanoides*; *B. crenatus* is rather more sensitive to copper but very much less sensitive to mercury. Further, the sensitivity of *B. balanoides* remains much the same over the range of

Table 15. *The effect of copper on* Balanus crenatus

Concentration of copper (mg./l.)	No. of barnacles tested	Preliminary examination—Numbers:				Apparent % surviving	Final examination nos. surviving	% survival
		Active	Moderately active	Slow movement	Inactive			
0	21	20	0	0	1	95	21	100
0·16	10	8	0	0	2	80	9	90
0·31	23	2	1	4	16	30	12	52
0·79	11	0	2	1	8	27	2	18
2·02	10	0	0	2	8	20	2	20

Exposure period, 6 hr.

Table 16. *The effect of mercury on* Balanus crenatus

Concentration of mercury (mg./l.)	No. of barnacles tested	Preliminary examination—Numbers:				Apparent % surviving	Final examination nos. surviving	% survival
		Active	Moderately active	Slow movement	Inactive			
0	50	36	0	14	0	100	50	100
2·3	23	0	0	19	4	83	9	39
3·1	17	0	0	13	4	76	4	24
4·1	20	0	0	17	3	85	3	15
6·7	28	0	0	8	20	29	0	0

Exposure period, 6 hr.

Table 17. *The effect of copper and of mercury on* Balanus balanoides *and* Balanus crenatus

Species	Poison	Median lethal concentration (mg./l.)			
B. balanoides	Copper	0·70	0·65	0·60	0·67
	Mercury	—	0·74	0·80	0·60
B. crenatus	Copper	1·6	0·52	0·45	0·45
	Mercury	?10·0	?3·0	2·2	2·2

Youngest ⟶ Oldest

Exposure Period, 6 hr.

ages examined—in particular, the youngest specimens have much the same sensitivity as the oldest, whereas the youngest specimens of *B. crenatus* are distinctly less sensitive to both poisons than older individuals, though the ratio between the two poisons remains roughly the same.

Extension of the exposure period to 24 hr., though it naturally affects the median lethal concentration, does not cause any marked differences in the relative effect of the two poisons. For this exposure period the median lethal concentration for copper for *B. balanoides* is 0·32 mg./l., and that for mercury 0·36 mg./l. The corresponding values for *B. crenatus* are 0·19 mg. Cu/l. and 1·35 mg. Hg/l.

DISCUSSION

The results described in this paper reveal considerable differences in sensitivity to copper and to mercury between different stages in the life history of one species and between corresponding life-history stages of the two species.

Thus, the naupliar stages of *B. balanoides* are highly sensitive to copper, the cyprid, at least immediately after it is formed from the sixth stage nauplius, is much less sensitive to this element. During the life period of the cyprid the sensitivity again increases, only to decrease markedly once settlement has occurred and metamorphosis begun; when metamorphosis is completed the sensitivity again increases. The sequence of changes for *B. crenatus* follows much the same general course, except that the cyprid of this species is so insensitive to copper that any differences in sensitivity between the free-swimming and the metamorphosing larva cannot be detected using natural sea water, and the sensitivity relationships of the adult *B. crenatus* differ from those of *B. balanoides*. Such differences emphasize the fact that the results which are obtained with one species cannot be applied without reservation to another, even if the two species are closely related.

The causes of these differences in sensitivity between stages and species are at present obscure. This obscurity is largely caused by the lack of knowledge of the effects of heavy metal poisons, and partly by marked differences in morphology and physiology between the various stages in the life history of a barnacle. There appear to be broadly two schools of thought on the mode of action of heavy metal poisons: one, that these elements never penetrate the tissues, but exert their effect by precipitating proteins on the surface of the animal and so interfering with such vital processes as respiratory exchange (Carpenter, 1927, 1930; Jones, 1947); the other, that heavy metals penetrate the tissues, where they act intracellularly as enzyme poisoners or protein precipitants. There is evidence (Clarke, 1947) which indicates that copper is taken up into the tissues of barnacles, and Mr W. R. Hunter (personal communication) has recently demonstrated that small amounts of copper cause a marked decrease in the oxygen uptake of *Marinogammarus marinus*, whereas small amounts of mercury have no significant effect on this process. This suggests that copper penetrates the tissues and has an effect on intracellular metabolic processes, particularly, it might be suggested, on the respiratory enzyme system. Though it is dangerous, without direct experimental proof, to suppose that these results obtained for *M. marinus* apply also to barnacles, it is reasonable to look for similar effects in the latter. In this connexion, the marked decrease in sensitivity to copper during metamorphosis is of particular interest since, if copper has an effect on the respiratory enzyme system and if respiration were anaerobic during metamorphosis, an explanation could be advanced for the decrease in copper sensitivity.

The effect of increasing copper concentrations in artificial sea water is a further point of interest. These effects may be due to the medium employed, but there are indications from the unpublished work just quoted that they are more likely to be due to the effects of the poison used. If, in these experiments, copper is acting as a direct poison and also affecting some metabolic process which in some way controls

the uptake of the poison, it is possible to interpret the results rather more fully. As has been mentioned earlier, copper may not only interfere with its own action, it may also affect the action of mercury when the two poisons are present together.

Measurement of the sensitivity to copper may also be a valid means of assessing the physiological state of a free-swimming cyprid, and this may prove a useful means of investigating the effect of variations in environmental factors on this stage. In particular, experiments have been begun on the effect of drying on the cyprid of *Balanus balanoides*, and the preliminary results of these experiments suggest that such studies may provide an explanation of the intertidal distribution of the adults of this species.

There is no reason to assume that variations in effect on intracellular processes are the sole causes of the differences in sensitivity recorded in this series of experiments. Penetration of the poison may be effected much more readily in one stage than in another. For example, in the naupliar stages an open gut is present, whereas in the cyprid the gut is closed. Penetration of poison may therefore take place more readily in the former than in the latter (Waterhouse, 1946, has demonstrated the presence of copper in the walls of the gut of *Lucilia cuprina* when fed on cultures containing this element), and the difference in sensitivity between the nauplii and the cyprids of both species investigated may be due largely to this cause.

Finally, apart from biological factors which make interpretation difficult, it is evident (Barnes & Stanbury, 1948) that the solution of either copper or mercury salts in sea water is a complicated process, and the existence of a number of complexes, the proportions of which may vary at different concentrations, adds another, and possibly an overriding factor to a situation already sufficiently complex if considered only in its biological aspects.

It is evident that too little is known, both of the effects and of the conditions of penetration of these poisons, fully to interpret the results described in this paper. More recent work, as yet unpublished, will take the interpretation a stage further.

SUMMARY

1. Experiments on the toxic effects of copper and of mercury on various stages in the life history, nauplii, cyprids, metamorphosing cyprids and adults of acorn barnacles are described. Most of the work has been carried out on the appropriate stages of *Balanus balanoides*, but it has been supplemented, wherever possible, by parallel experiments using *B. crenatus*.

2. Cupric sulphate and mercuric chloride solutions were used as sources of the poisons, and the actual concentration of poison present was determined at the end of each experiment. The period of exposure to the poison was usually 6 hr.

3. A sharp decrease in sensitivity occurs, both in *B. balanoides* and *B. crenatus*, between the last naupliar stage and the cyprid. The relative sensitivity of the cyprid of *B. balanoides* to copper and to mercury is similar to that of the sixth stage nauplius, but the cyprid of *B. crenatus* is particularly insensitive to copper, but is more sensitive to mercury than that of *B. balanoides*.

4. Cyprids of *B. balanoides* only settle in the laboratory some 4 or 5 days after being taken in the plankton and during this period the sensitivity to copper and mercury increases. Settlement can be prevented by very low concentrations of copper and mercury, even though there are no obvious lethal effects.

The cyprid of *B. crenatus* settles more readily in the laboratory, and little change in sensitivity seems to occur during the short interval between catching and settlement.

5. Metamorphosis of the cyprid of either *B. balanoides* or *B. crenatus* cannot be prevented by the concentrations of copper (up to 7 mg./l.) possible in sea water. This means that another sharp change in sensitivity occurs after the cyprid of the former species has settled but, because of the low sensitivity of the free-swimming cyprid of *B. crenatus*, such a change has not been detected for this species.

6. The young barnacle of both species immediately after metamorphosis is much more sensitive to copper than the metamorphosing cyprid. For *B. balanoides* the sensitivity, both to this poison and to mercury does not change significantly as the barnacle grows, but a small specimen of *B. crenatus* (less than one month old) is appreciably less sensitive, both to copper and to mercury, than slightly older individuals.

7. Copper and mercury appear to be roughly equi-toxic to the adult of *B. balanoides*; the adult *B. crenatus* is slightly more sensitive to copper and distinctly less sensitive to mercury than that of *B. balanoides*.

8. In view of these differences in sensitivity of the various stages in the life history of two closely related species, the results obtained with one species should not be held to apply to other species of barnacles.

9. A number of other experiments were carried out using the free-swimming cyprids of *B. balanoides*. Diluted sea water, though it has no toxic effect in itself over short exposure periods, markedly reduces the toxicity of both copper and mercury. Hypertonic sea water also reduces the toxicity of copper. The sensitivity of these larvae to either copper or mercury is not affected by the presence of sodium oleate.

Exposure of these larvae to a wide range of copper concentrations in artificial sea water reveals certain anomalies which may be important in explaining the results obtained when they are exposed to mixtures of copper and mercury in natural sea water. The toxic effect of mercury seems to be similar in either natural or artificial sea water.

The authors wish to record their thanks to the Marine Corrosion Sub-Committee for their permission to publish this work, and they also wish to acknowledge the help and encouragement they have received from Prof. J. E. Harris. The senior author is also much indebted to his colleagues, Mr M. W. H. Bishop and Mr W. R. Hunter for their help in the later stages of this work.

REFERENCES

Barnes, H. (1946). *J. Mar. Biol. Ass. U.K.* **26**, 303.
Barnes, H. & Stanbury, F. A. (1948). *J. Exp. Biol.* **25**, 270.
Carpenter, K. E. (1927). *Brit. J. Exp. Biol.* **4**, 378.
Carpenter, K. E. (1930). *J. Exp. Zool.* **56**, 407.
Clarke, G. L. (1947). *Biol. Bull. Woods Hole*, **92**, 73.
Cole, W. H. (1932). *J. Exp. Zool.* **63**, 143.
Harris, J. E. (1947). *J. Iron Steel Inst.* 1946, 297.
Hykes, O. V. (1931). *C.R. Soc. Biol., Paris*, **106**, 328.
Ishida, S. (1936). *Sci. Pap. Inst. Phys. Chem. Res. Tokyo*, **30**, 195.
Jones, J. R. E. (1935). *J. Exp. Biol.* **12**, 165.
Jones, J. R. E. (1937). *J. Exp. Biol.* **14**, 351.
Jones, J. R. E. (1938). *J. Exp. Biol.* **15**, 394.
Jones, J. R. E. (1939). *J. Exp. Biol.* **16**, 425.
Jones, J. R. E. (1941). *J. Exp. Biol.* **18**, 153.
Jones, J. R. E. (1947). *J. Exp. Biol.* **23**, 298.
Löhner, L. (1924). *Pflug. Arch. ges. Physiol.* **203**, 524.
Ludwig, W. (1927). *Z. vergl. Physiol.* **6**, 623.
Miller, M. A. (1946). *Biol. Bull. Woods Hole*, **90**, 122.
Müller, F. M. (1940). *Arch. neerl. Zool.* **4**, 113.
Powers, E. B. (1917). *Illinois Biol. Monogr.* **4**, 3.
Pyefinch, K. A. (1948*a*). *J. Mar. Biol. Ass. U.K.* **27**, 451.
Pyefinch, K. A. (1948*b*). *J. Mar. Biol. Ass. U.K.* **27**, 464.
Visscher, J. P. (1928). *Biol. Bull. Woods Hole*, **54**, 327.
Waterhouse, D. F. (1946). *Bull. Coun. Sci. Industr. Res. Aust.* no. 191.

THE THEORY OF THE FOVEA

By R. J. PUMPHREY

From the Zoological Laboratory, University of Cambridge

(*Received* 20 *April* 1948)

(With Ten Text-figures)

INTRODUCTION

There is ordinarily in the retina of vertebrates with good diurnal vision a region where the density of cones is higher than elsewhere. To this region the name *area* (or less appropriately *area centralis*) has been applied. In anthropoid anatomy, it is more often referred to as the *macula* or yellow spot, because the region of high cone-density is more or less coextensive with the distribution of a yellow pigment which is conspicuous on ophthalmoscopic inspection of the retina. This *area*, because of the high cone-density and because it is usually situated near the optic axis where the effect of aberration is least, is believed to be a region of high resolving power.

Within the *area* there is very frequently a *fovea*, so called because it appears as a depression or pit when the retina is viewed from the pupil side. Histologically the *fovea* is a region of higher cone-density than the rest of the *area* and the density rises to a maximum at its centre. Moreover, it is established that a one-to-one relation exists between the cones of the *fovea* and the ganglion cells, i.e. each cone is individually represented by a fibre in the optic nerve. It is quite probable, however, that this one-to-one correspondence extends well out into the *area* if not over the whole of it.

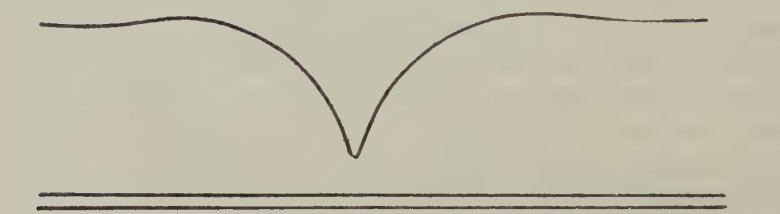
Both *area* and *fovea* are clearly part of the equipment of the eye for daylight vision. Rods are scarce in the *area* and invariably absent from the *fovea*, except in one or two scattered groups of animals in which nocturnality is evidently a relatively recent secondary acquisition (owls, *Sphenodon*) and the *fovea* appears degenerate.

The depression on the inner aspect of the retina from which the *fovea* takes its name is caused by the displacement radially from its centre of the cells of the neural layers of the retina, leaving a cavity filled by the vitreous humour. And *foveae* are divisible into two fairly distinct classes on the basis of the form of this depression. In one class the depression is relatively shallow and saucer-shaped; in the other it is deep and funnel- or whirlpool-shaped. The shallow class includes the *fovea* of anthropoids, and also the temporal *fovea* of some birds. The *foveae* of fish, reptiles and birds (with the exception mentioned) belong to the deep class which Walls has labelled convexiclivate (Fig. 1).

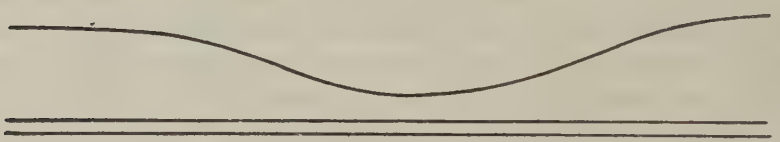
Within the classes there is a considerable variation of form and the anthropoid type is often rather irregular in outline. But it is a consistent feature of the latter that the central floor of the fovea (apposed to the region of highest cone-density) is relatively flat with a low curvature; whereas in the convexiclivate type the curvature

at the centre is relatively very high. Consequently, if any functional significance attaches to the *shape* of the fovea, it is rather improbable that it will be the same for the two classes.

Walls (1937) appears to have been the first to attempt a specific functional interpretation of the shape of the convexiclivate fovea. It had previously been supposed that the 'purpose' of the radial displacement of the neural layers away from the centre was simply to clear the optical path between pupil and cones at the point where the cones were densest and visual acuity was potentially highest. Walls correctly pointed out that the neural retina in life was optically homogeneous and its transparency not measurably different from that of the vitreous, so that the advantage to be gained by substituting one for the other over a few per cent of the total optical path between pupil and cones was vanishingly small. The extreme regularity of the profile of the convexiclivate fovea suggested to him a lens-like



Convexiclivate type



'Anthropoid' type

Fig. 1.

function. Refraction must occur at the vitreo-retinal boundary if the refractive indices of vitreous and retina are not identical; and that they are not can be inferred from the fact that the fovea can be seen by ophthalmoscopic inspection in the living retina. Walls (1940) later resuscitated measurements of the refractive indices of vitreous and retina by Valentin showing that the refractive index of the retina was the higher in the ratio of about 1·006. And on this basis he computed by a graphical method the 'magnifying effect' of the fovea. From this result, he arrived at an estimate of the extent to which 'visual acuity' was improved by foveal refraction. Later (1942, p. 662) he states that '*foveally* the visual acuity of some hawks and eagles reaches a value at least eight times that of man'.

Walls is entitled to great credit for seeing that a problem existed and for his courageous attack upon it. But his analysis, especially in its neglect of aberration, is incomplete and his later conclusions are in error. It is the purpose of this paper to attempt a more thorough assessment of the effect of foveal refraction and to suggest a probable function for the convexiclivate fovea.

REFRACTION BY THE FOVEA

The central fovea of Falconiform birds is typically convexiclivate and of great regularity and symmetry. Walls based his analysis on the central fovea of a buzzard. The following treatment is based on Polyak's (1941) figure of the central fovea of the golden eagle (*Aquila chrysaëtus*) which is very closely similar in contour. In Walls's figure of the buzzard's fovea the external limiting membrane is appreciably curved. In the eagle this membrane (which may be taken to be the locus of the seen image) is very nearly plane over the whole foveal area. And this fact somewhat simplifies the geometry of the problem.

Fig. 2 is a reproduction of Polyak's figure. And it can be seen that, except very near to the centre where the curvature changes sign, the profile can be represented by quadrants of circles which are tangent and whose centres lie in the external limiting

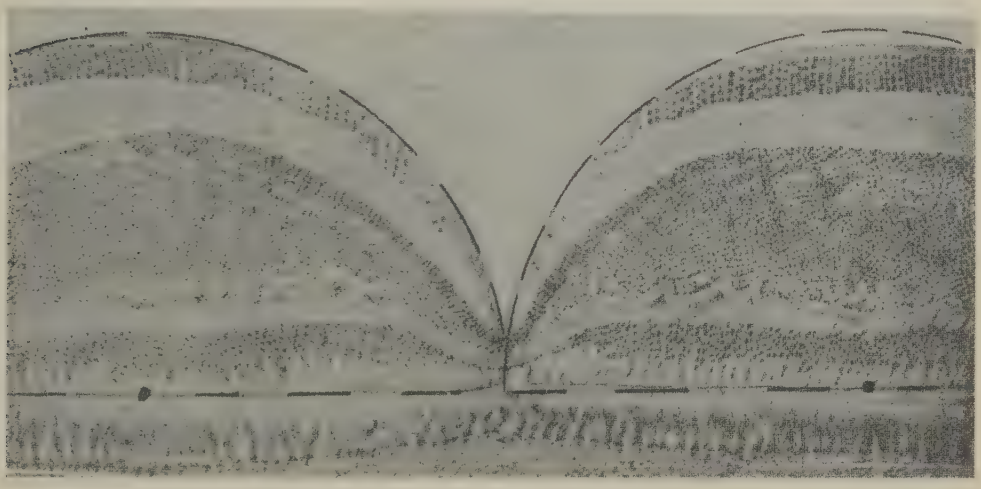


Fig. 2. Reproduction of section across the centre of the central fovea of *Aquila* from Polyak (1941). Superimposed are a dashed line in the plane of the external limiting membrane, and circles centred on this line (see text).

membrane. The slight divergences are probably attributable to unequal post-mortem shrinkage which may also be responsible for the small tear in the external limiting membrane near the centre. In Walls's figure of the *Buteo* fovea the coincidence with circles is even more exact, so it is reasonably safe to assume that such a profile represents with considerable accuracy the *in vivo* configuration of the fovea in Falconiform birds. As to the geometrical form at the centre it is evident by inspection that a paraboloid of revolution about the axis of symmetry is a reasonable approximation. It will be seen that no more explicit assumption is necessary.

This relatively simple geometrical form makes it possible to compute the effect of refraction with substantially greater accuracy than is possible by graphical methods. And it will be possible to show that in certain respects it is the 'best' form.

For the calculations which follow, radial symmetry is assumed and dimensions as indicated in Fig. 3. It is also assumed, following Valentin, that the ratio of refractive indices of retina and vitreous is 1·0063.

Now consider the path of single ray PQ normal to the plane of the photoreceptor cells until refracted at Q to intersect this plane at S. Let x be the distance between PQ and OY, the axis of symmetry of the fovea, and let X be the distance OS. The effect of refraction is evidently to displace the point of intersection with the image plane radially from the axis of the fovea by a distance $(X-x)$.

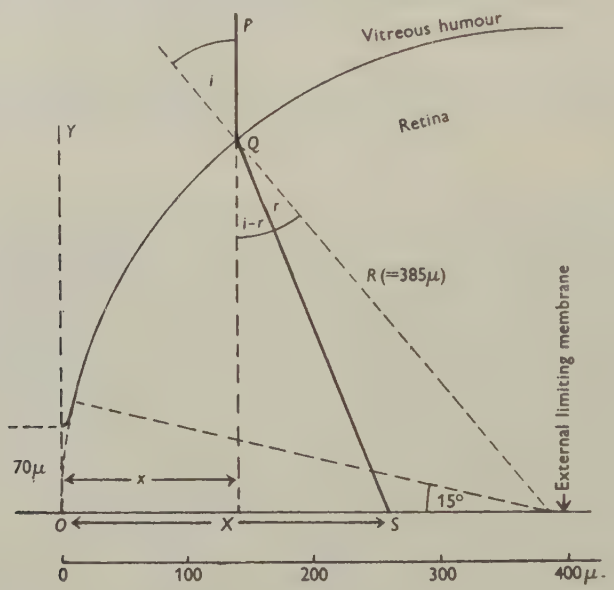


Fig. 3.

Suppose the ray PQ to trace out a cylinder of radius x about the foveal axis OY. Then the locus of the intersection of the ray with the image plane will be a circle, radius X, whereas, but for refraction, it would have been a circle, radius x; and the circumferences of these circles will also be in the ratio X/x. Hence, a magnification factor in the tangential direction can be defined, say

$$M_t = \frac{X}{x}.$$

Similarly, a radial magnification factor M_r can be defined. Evidently

$$M_r = \frac{dX}{dx}.$$

The physical meaning of these factors can be seen by considering the set of narrow pencils of rays which, without refraction, would form in the image plane a small circle of diameter δ and centre distant x from OY ($\delta \ll x$). Refraction will cause the set to intersect the image plane in an ellipse, whose centre is distant by $M_t x$ from OY and whose axes are in the tangential and radial directions (with respect to the centre of the fovea) and are equal to $M_t\delta$ and $M_r\delta$ respectively.

The values of these factors can be readily evaluated for values of x between 10 and 385μ. Let i be the angle of incidence, r the angle of refraction, R the radius of curvature, and μ the ratio of refractive indices of neural retina and vitreous humour, then

$$\sin i = \mu \sin r,$$

and from Fig. 3

$$x = R(1-\sin i),$$

$$X = x + R\cos i \tan(i-r).$$

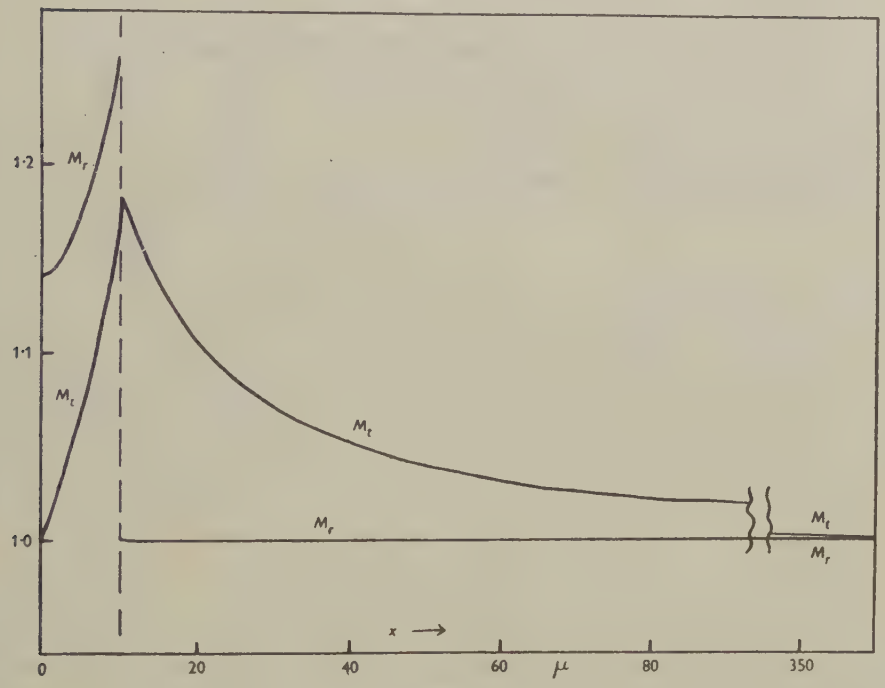


Fig. 4.

Hence

$$M_t = \frac{X}{x} = 1 + \frac{\cos i}{1-\sin i}\tan(i-r)$$

and

$$M_r = \frac{dX}{dx} = 1 + \tan i \tan(i-r) - [\sec^2(i-r)]\left[1 - \frac{\cos i}{\mu \cos r}\right]$$

$$\doteq \tan i \tan(i-r) + \frac{\cos i}{\mu \cos r}.$$

The resulting values for M_t and M_r are plotted in Fig. 4, to the right of the dashed line which corresponds to the value of x where the curvature changes at an angle of incidence of 75°. As x decreases, M_t rises from unity to about 1·2. M_r is practically unity for all values of x. It rises from 0·994 ($= 1/\mu$) at the periphery to 1·000 in the immediate vicinity of the point of inflexion.

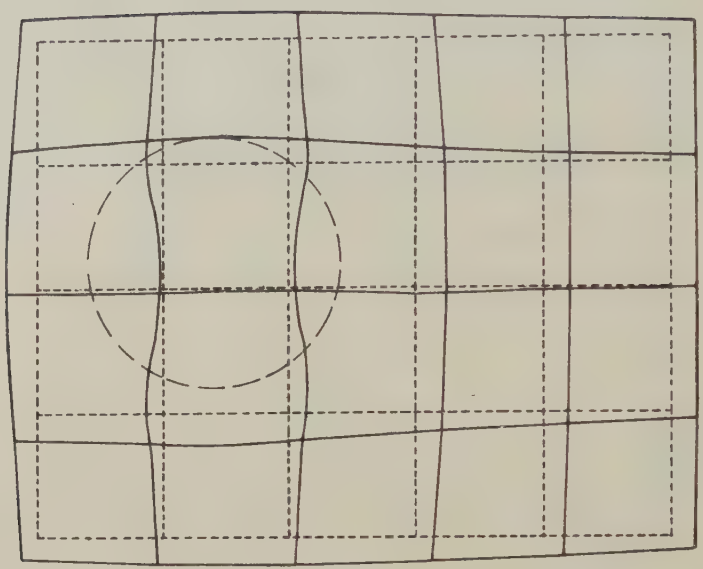


Fig. 5. The solid lines represent the effect of foveal refraction on an image which would otherwise have the form shown dotted. The circle indicates the centre of the fovea, its radius is 10 μ.

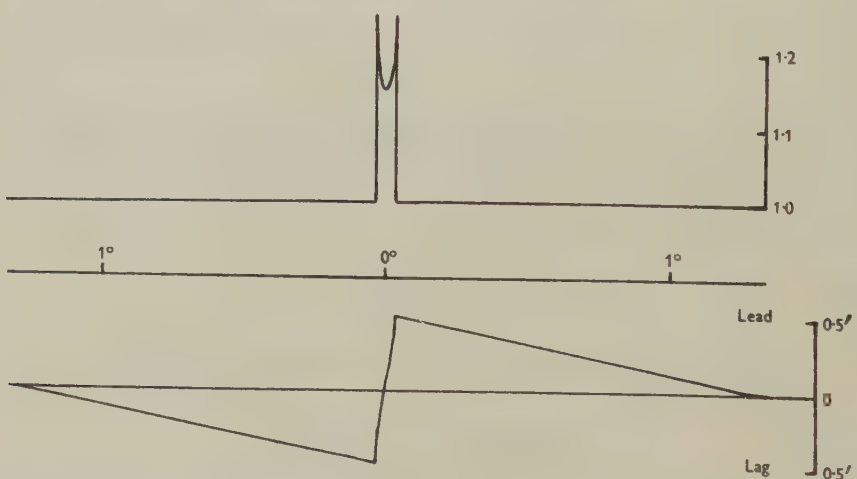


Fig. 6. The upper curve shows the ratio of the angular velocities of image (M,θ) and of object (θ). The lower shows the angular displacement of the image from its 'true' position. The horizontal co-ordinate expresses the angle between object and axis of symmetry of the fovea (focal length of eye assumed to be 17 mm.).

It is now necessary to consider the effect of the change of curvature in the centre of the fovea. It is assumed that the profile is here a parabola, $y = ax^2 + c$, say. Then, using the same notation as previously,

$$\sin i = \mu \sin r, \quad x = \frac{\tan i}{2a}, \quad X = x + y \tan (i - r),$$

$$M_t = \frac{X}{x} = 1 + (\tfrac{1}{2} \tan i + 2ac \cot i) \tan (i - r),$$

$$M_r = \frac{dX}{dx} = 1 + \tan i \tan (i - r) + (\tfrac{1}{2} \sin^2 i + 2ac \cos^2 i) [\sec^2 (i - r)] \left[1 - \frac{\cos i}{\mu \cos r}\right].$$

From Fig. 3, $y = c = 70\mu$ when $x = 0$, and a can be found if the parabola is assumed to be tangent to the circle at the point where the curvature changes ($i = 75°$, $x = 10\mu$). Evaluating M_t and M_r for $x < 10\mu$ we find the values shown in Fig. 4 to the left of the dashed line. They are uncertain to the extent that the exact form of the central depression is uncertain. But there is no uncertainty about the existence of an abrupt change of slope in the curve for M_t and of an abrupt change in the magnitude of M_r where the curvature changes sign.

The effect of the changes in these factors with distance from the centre of the fovea will perhaps be clearer from a consideration of Fig. 5 which shows the effect which foveal refraction would have on a rectilinear pattern (aberration and diffraction being neglected). Another way of regarding the effect is to imagine a point object moving across the visual field in such a way that its image traverses the centre of the fovea. If the angular velocity of the object is θ the angular velocity of the image is $M_r\theta$; and the image will lag behind the object till the centre is reached and thereafter lead it (see Fig. 6).

ABERRATION AND DIFFRACTION

So far attention has been limited to single rays and narrow pencils and it has been assumed that point images can exist. But, in fact, a convergent beam of fairly wide angle is required to form from a point source an image in which the central bright zone is not large compared with the area of a single cone. And this is evidently a necessary condition if full use is to be made of the high cone-density of the fovea in the resolution of detail in the visual field. The angle can be calculated directly from a knowledge of the cone-density or rather less directly from a comparison of the relative sizes of human and avian foveal cones and a knowledge of the pupil width below which diffraction limits the acuity of the human eye.

The cone-density at the centre of the fovea of a buzzard is stated by Rochon-Duvigneaud (quoted by Walls, 1942) to be 10^6/mm.^{2} so that the area of a single cone is approximately $1\mu^2$. For two point images to be resolvable by Rayleigh's criterion the angular width of the convergent pencils forming them must be greater than ϕ, where

$$\tan \tfrac{1}{2}\phi = \frac{0{\cdot}61\,\lambda}{\mu r},$$

and λ = wave-length *in vacuo*, μ = refractive index of the medium, and r = distance between the centres of the images.

If we suppose the cones to be packed as a regular honeycomb, the distance between the centres of adjacent cones will be 1·07, and the least distance between the centres of cones which do not touch will be 1·86μ. It is probable that for two images to be just recognizably separate, their distance apart will be between these values; hence r is put at 1·5μ. The wave-length of light of maximum photopic luminosity is $5{\cdot}6 \times 10^{-5}$ cm. and the mean refractive index of the ocular media is about 1·33.

Hence $$\tan \tfrac{1}{2}\phi = 0{\cdot}17, \quad \phi \doteqdot 19^\circ.$$

Alternatively, accepting that diffraction limits the acuity of the human eye for pupil widths less than 2·3 mm. (Schlaer, Smith & Chase, 1941), taking the cone-density at the centre of the human fovea to be 200,000 per mm.^{2} (one-fifth of the avian density, Walls 1942) and assuming a similar focal length for the avian and human eyes (which is probably true for the larger hawks)

$$\phi_{\text{avian}} = \sqrt{5}\,\phi_{\text{human}} \doteqdot \sqrt{5}\tan^{-1}\left[\frac{2{\cdot}3 \text{ mm.}}{\text{posterior nodal distance (17 mm.)}}\right] \doteqdot 17^\circ.$$

The agreement is fair, but to be on the safe side the value $\phi = 15^\circ$ will be assumed. Now it can be seen from inspection of Fig. 7 that a pencil of rays which, in the absence of the fovea, would converge to a point in the image plane at an angle of 15°, will include part of the zone of inflexion if its axis is less than 20μ from the axis of the fovea. And if any part of this zone is included aberration is severe. For simplicity, consider the special case of a 15° pencil coaxial with the fovea. The peripheral rays of this pencil encounter the retina at an angle of incidence approximately 81° and will consequently be refracted outwards about 2°. The effect of foveal refraction is, therefore, to replace the point image in the plane of the external limiting membrane by a circle of confusion embracing over thirty cones.

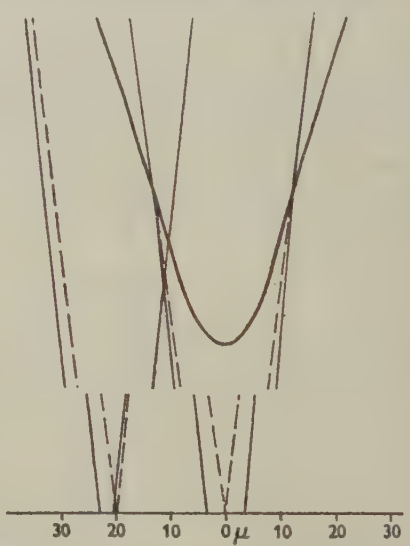


Fig. 7. The effect of refraction on two 15° pencils of rays centred 20μ from the axis and on the axis respectively. The heavy line represents the profile of the vitreo-retinal boundary. The light continuous lines represent the course of peripheral rays of the pencils and the dashed lines their course if there had been no refraction. The discontinuity indicates the omission of 40μ in the vertical direction.

This degree of aberration could be reduced by a reduction of pupil width, but only at the cost of increasing the extent of the diffraction pattern. By no compromise could the bird utilize the high cone-density at the centre of the fovea to improve its acuity as compared with man.

On the other hand, outside the zone of severe aberration (radius 20μ about the axis) the magnification by the fovea is small and, as we have seen, in the tangential direction only; in the radial direction images

are actually very slightly reduced. It is not evident that such a characteristic could aid in increasing acuity, though perhaps it does little to diminish it.

DISCUSSION

In accepting the idea that, so far from increasing acuity, foveal refraction has little effect at the periphery and very substantially diminishes the potential acuity in the centre we are forced to look for an alternative function which makes the sacrifice worth while.

The convexiclivate fovea is just such a device as one would choose to aid in the exact alignment of an optical system, for it has the remarkable property of transforming a radially symmetrical image into an asymmetrical one except when there is exact coincidence between the axes of symmetry of the fovea and of the object. (This is evident from Fig. 5.) This strongly suggests that the convexiclivate fovea is concerned with two cognate functions, the maintenance of accurate fixation and the sensitive appreciation of angular movements of a fixated object.

In assessing the correctness of this conclusion it is important to realize that the efficiency of such a device for detecting and correcting misalignment does not (like acuity) depend on the completest possible resolution of detail in the image. When a diatom is examined under a high power of the microscope the symmetry or lack of symmetry in the sculptured pattern is often *more* evident if the full aperture is not used or if the diatom is slightly out of focus. The condition for its best appreciation is not that in which the image most exactly represents the object, but that in which the effects of diffraction and aberration combine to give maximum contrast between one element of the image and another. If the object approaches the size of a single point, its image, viewed through an ordinary microscope, will be radially symmetrical for all combinations of lack of focus and small aperture. But with an ocular having the attributes of the convexiclivate fovea, there will only be radial symmetry for the images of points lying on its axis. Obviously the asymmetry in the image of a point not quite on the axis will be most readily perceived if the image is large enough for its form to be appreciated and does not itself approach a point, i.e. if it is aberrated or out of focus or the aperture is so small that the diffraction pattern is readily seen.

This argument has been emphasized for two reasons. In the first place, it makes clear that though in a large measure the optical structure conducing to high acuity is identical with that conducing to a high capacity for fixating objects in the visual field, yet there is a level of refinement at which acuity must gain at the expense of accuracy of fixation and of movement sensitivity or vice versa. In the second place, the argument casts doubt on the validity of acuity estimates based on the optomotor reaction; for it clearly does not follow, as has been frequently assumed, that because an animal has responded to a moving visual field its eye has resolved the field as a microscope under optimal conditions resolves the detail of *Pleurosigma*. All that can be legitimately inferred is that the visual field has somewhere in the photosensitive zone produced a gradient of light intensity which is above the differential threshold of the photosensitive elements. For example, an interferometer can be used to register changes in the distribution of light in a field with

extraordinary accuracy but it does not 'resolve' it, i.e. there is no simple point-to-point correspondence between the field and what is seen in the interferometer.

The convexiclivate fovea then is an appropriate device for emphasizing angular displacements; and the same properties make it suitable for increasing the animal's awareness of angular movements. Both phenomena depend on an important and almost wholly mysterious property of the vertebrate optical system, its extreme sensitivity to trifling irregularities in an otherwise regular pattern. This property lies at the root of a number of well-known optical illusions and also of the extraordinary Vernier acuity of the human eye. If two lines are parallel and placed end to end (like the lines of a Vernier scale) they can be seen to be misaligned when their angular separation is very much less than the angular separation of two just resolvable points.

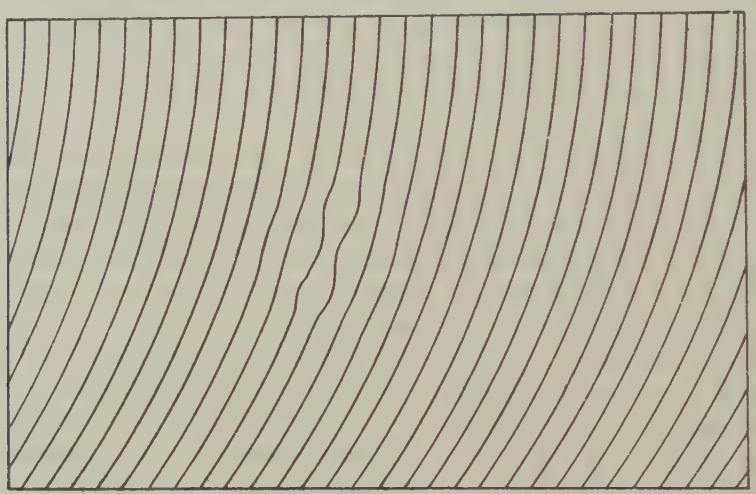


Fig. 8. Distortion by the fovea. The lines represent the successive images at equal time intervals of the boundary of a regular object when the object moves steadily across the visual field. If this picture is viewed at 7 m., the area of irregularity subtends an angle about equal to the angle subtended by the central part of the hawk fovea. It will be found that the irregularity is very evident to the human eye at this distance though the lines are resolvable with difficulty.

The eye, therefore, cannot be resolving the discontinuity at the junction of the lines; and what is seen as misalignment must be the lack of straightness in the single line which the two lines combine to form. Vernier acuity is in fact a particular example of sensitivity to regularity and irregularity of form and pattern. It has nothing to do with acuity in its ordinary sense, though, of course, acuity must be adequate to resolve the general structure of the pattern. If the lines, in the example quoted, could barely be distinguished from the background, obviously their misalignment could not be so well appreciated.

Vernier acuity has not been directly demonstrated in animals, but birds have been shown by ingenious methods to be subject to optical illusions similar to those of man and it is reasonable to assume that they have a basically similar visual sensitivity for patterns.

The effect of the convexiclivate fovea of the hawks on the image of a regular object passing across it is to introduce momentarily the kind of irregularity which we might expect to be most conspicuous, namely a small and fluctuating irregularity in an otherwise regular image. The essential point here would seem to be the emphasis given to the distortion by its juxtaposition to almost undistorted parts of the image (Fig. 7) and for this the abrupt discontinuity in the refraction of the central and peripheral parts of the fovea is important. Outside the central zone of aberration, the distortion is very small, no image point being displaced from its 'true' position by more than $2\frac{1}{2}$ cone diameters. And the central zone contains only about 1000 out of the total of roughly 500,000 cones in the fovea. Over more than 95% of the area of the fovea, therefore, almost the full potential acuity can be realized.

It will have been noted that the conditions for optimal fixation of a limitingly small object and for optimal detection of angular movement of a larger one, though similar, are not quite identical. For the former it is only necessary that misalignment shall be associated with distortion of an aberrated image. There is not the same requirement for freedom from distortion in the immediately surrounding area.

It seems probable that fixation of objects and appreciation of angular movement were primitive functions of the optical system, and that the power of resolving detail, at first a useful accessory, grew in relative importance as central functions, such as recognition of and memory for visual patterns, emerged. Consequently, it is not surprising that the most primitive foveae (in fish) are of moderately convexiclivate form. Ultimately, as we have seen, there must be rivalry between acuity and movement sensitivity, especially for limitingly small objects; and two directions of development are possible. The first is to specialize the centre of the fovea as an alignment device relying for acuity on the periphery of the fovea and the parafoveal area. The second is to sacrifice the higher development of the alignment function and devote the centre of the fovea to the optimal resolution of an undistorted image.

The reflexion of these developments on the geometry of the fovea is possibly indicated by such a scheme as in Fig. 9.

The scheme is not to be regarded as strictly geneological, but merely illustrative of possible lines of functional development. The central fovea of the hawks represents the highest development of the fovea as a fixation device which is consistent with the realization of nearly maximal acuity and movement sensitivity. The kingfisher has sacrificed acuity and movement sensitivity to fixation, for aberration at the centre is greater, the zone of aberration is more extensive and the transition to the zone of negligible distortion is less abrupt than in the hawks (see Fig. 10). Another possible development is illustrated by the fovea of the flamingo, shearwater, herring gull, curlew, snowgoose, cormorant, birds in which, according to Wood's (1917) figures, both area and fovea are more or less elongated along the horizontal equator of the eye. Such a design appears suited to fixation of the horizon and to effecting a preferential increase in sensitivity to vertical movements of objects in relation to the horizon (Pumphrey, 1948). All these birds frequent open spaces of land or water.

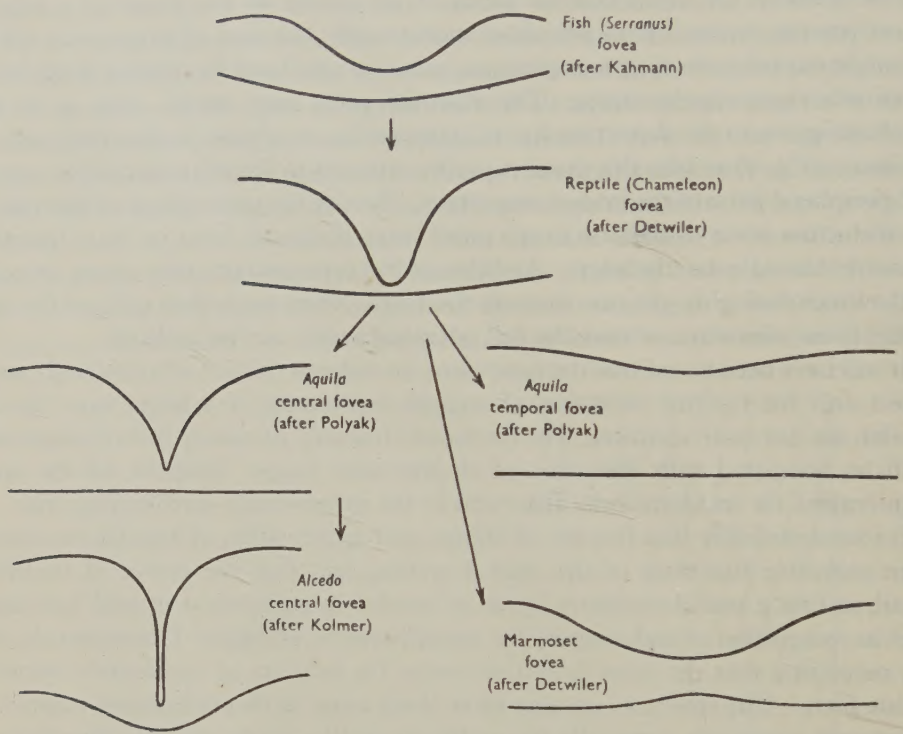


Fig. 9.

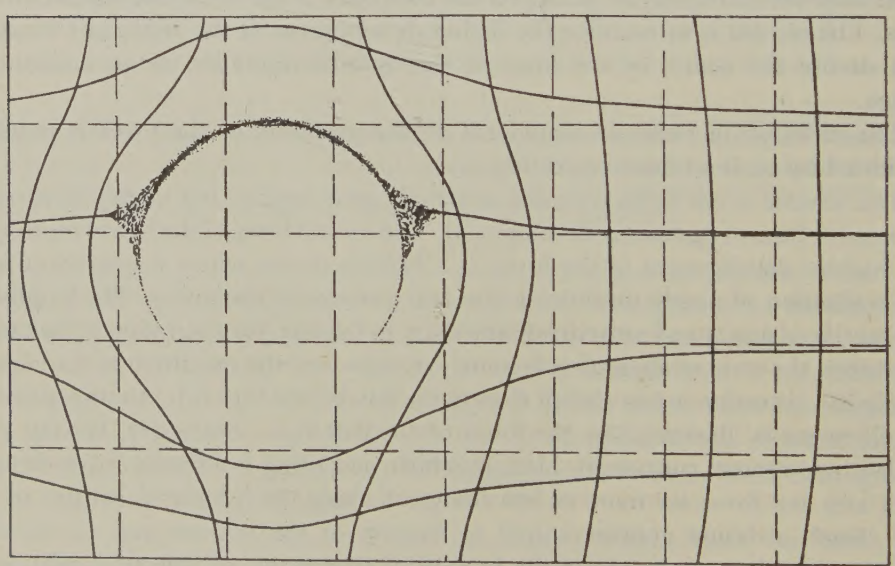


Fig. 10. Distortion of a rectangular pattern by the central fovea of the kingfisher for comparison with Fig. 5. As Kolmer gives no scale it has been assumed that the fovea is about one-half the size of the eagle's. The dashed squares have a side of about 5 μ.

It seems very significant that the shallow 'anthropoid' type of fovea is found *only* where the eyes are used for binocular vision, and where central superposition or fusion of the left and right foveal images must be presumed to occur. It may well be that in these circumstances the optical errors inseparable from a steep foveal clivus cannot be tolerated. Distinct central (monocular) and temporal (binocular) foveae occur in the retinae in many distantly related birds (mostly predators) which are capable of occasional binocularity. It cannot yet be said with certainty that in these the temporal fovea is always more 'anthropoid' than the central, but it is evidently the case for the eagle, and Kolmer (1924) states that the temporal fovea of the kingfisher is more open than the central, though he does not figure it.

Birds, as a rule (even those with well-developed binocular vision), appear to scrutinize distant objects monocularly using the central fovea if they have one. We have seen reason to believe that no exceptionally high acuity is associated with the central fovea, but it is, nevertheless, alleged that birds have exceptionally 'acute' vision as compared with man. On the basis of cone-density alone it is possible that some birds have an acuity from two to three times that of man. To realize even this figure needs a large eye with a very wide aperture and consequently a corresponding degree of chromatic aberration in the dioptric system (quite apart from the foveal contribution). And no trick can wholly overcome this disability except at the sacrifice of colour vision which contributes to the resolving power actually attainable in the field.

But there is, in fact, no evidence of very high acuity in birds which cannot be interpreted either as an exceptional sensitivity to movement of an object scrutinized, or to an exceptional ability to 'hold on' to an object once seen. And these faculties, as we have seen, are just those which a convexiclivate fovea might be expected to aid substantially. Man, with his flat fovea, finds it extremely hard to continue to hold in sight an object which is approaching the limit of visibility, and, of course, once lost the chances of finding it again are small. It is likely that a device which would keep the object accurately centred would effect an astonishing increase in the range to which a bird or an aircraft could be visually followed by a human observer.

SUMMARY

The structure and distribution of the two principal types of fovea are briefly described. Using the convexiclivate central fovea of the eagle as a type the effect of refraction at the boundary of retina and vitreous humour on the foveal image is computed. It is shown that, when aberration and diffraction are taken into account, no improvement in acuity can result from refraction at the fovea. On the contrary, the results strongly suggest that the convexiclivate fovea is a device for achieving improved fixation and improved sensitivity to movement of objects in the visual field at some sacrifice of acuity.

The application of these results to other variants of fovea is discussed, and a scheme indicating possible relations between function and geometrical form is proposed.

REFERENCES

DETWILER, S. R. (1943). *Vertebrate Photoreceptors.* New York.
KAHMANN, H. (1934). Über das Vorkommen einer Fovea centralis im Knochenfischauge. *Zool. Anz.* **106**, 49–55.
KOLMER, W. (1924). Über das Auge des Eisvogels. *Pflüg. Arch. ges. Physiol.* **204**, 266–74.
POLYAK, S. L. (1941). *The Retina.* Univ. of Chicago Press.
PUMPHREY, R. J. (1948). The sense organs of birds. *Ibis*, **90**, 171–99.
ROCHON-DUVIGNEAUD, A. (1919). Quelques données sur la fovea des oiseaux. *Ann. Oculist., Paris*, **157**, 673.
SHLAER, S., SMITH, E. L. & CHASE, A. H. (1941). Visual acuity and illumination in different spectral regions. *J. Gen. Physiol.* **25**, 326–33.
WALLS, G. L. (1937). Significance of the foveal depression. *Arch. Ophthal., N.Y.*, **18**, 912–19.
WALLS, G. L. (1940). Postscript on image expansion by the foveal clivus. *Arch. Ophthal., N.Y.*, **23**, 831–2.
WALLS, G. L. (1942). *The Vertebrate Eye.* Michigan: Cranbrook Inst. of Science.
WOOD, C. A. (1917). *The Fundus oculi of Birds.* Chicago: Lakeside Press.